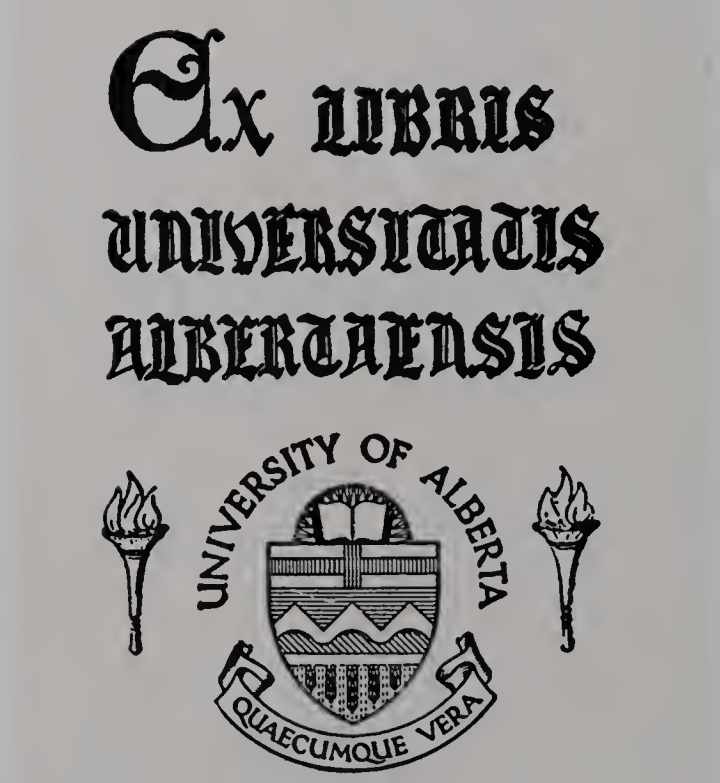

THE UNIVERSITY OF ALBERTA

IN VITRO STUDIES ON LEUKOCYTES

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "In vitro Studies on Leukocytes," submitted by William Thompson Howson, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

ABSTRACT

In vitro lymphocyte stimulation was demonstrated in mixtures of leukocytes derived from chickens having different histocompatibility alleles at the B locus. However, unsolved problems of technique precluded its use for studying this phenomenon. A comparison was made with similar cultures of human leukocytes. The origin of the plasma used in the medium was shown to affect the success of chicken leukocyte cultures.

A study was made on a number of hormones to determine their significance for this experimental technique. For this purpose stimulation by phytohemagglutinin was used as a model of antigenically induced proliferation of human leukocytes. Using approximately normal plasma levels, progesterone and 17-β-estradiol had no significant effect whereas hydrocortisone and human chorionic gonadotropin profoundly reduced lymphocyte proliferation.

The variables associated with isoantigenic stimulation *in vitro* and the possible mechanisms of action of some of these hormones were discussed.

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INTRODUCTION AND GENERAL LITERATURE REVIEW

Beginning in the 1920's many tissue culture studies have been carried out on peripheral blood cells. Avian blood cultures characteristically came to be dominated by proliferating macrophages (Carrel *et al.*, 1922), whereas similar mammalian cultures showed little (Maximow, 1928, cited in Jacoby, 1965, p. 50; Chrustschoff, 1935) or no (Caffier, 1928, cited in Jacoby, 1965, p. 50) proliferation of any cell type.

Little subsequent progress was made in obtaining further proliferation of mammalian blood cells until the introduction of a technique by Osgood *et al.* (1955) who attributed this success to the provision of adequate growth conditions through the deliberate formation of metabolic gradients (e.g. oxygen tension) within the culture.

However, Nowell (1960a) found that these gradients had little effect on mitosis and that the mitotic stimulus was due to a substance derived from *Phaseolus vulgaris*, called phytohaemagglutinin (PHA), which had been used to facilitate the separation of erythrocytes from the leukocytes prior to culturing.

Nowell (1960b) claimed that the large cell type which underwent mitosis (hereafter to be referred to as a hemocytoblast) was derived from monocytes and large lymphocytes whereas MacKinney *et al.* (1962) suggested that it was derived exclusively from lymphocytes. The latter interpretation has been firmly supported by Marshall (1963) and Rabinowitz (1964).

The next important finding was made by Permain et al. (1963) who, in attempting to prove that PHA was acting as an immunological stimulus, showed that PPD (purified protein derivative from Mycobacterium tuberculosis) would also induce mitosis in lymphocytes but only in those derived from individuals sensitized to PPD. Shortly afterward, Elves et al. (1963) showed a similar specific effect with a variety of antigens.

Although the effect of PHA suggests its activity as an antigen, fundamental differences obscure its relationship to what are considered normal antigens. It is well established that normal antigens act as mitotic inducing agents only on cells derived from individuals precisely sensitized to the antigen; on the other hand PHA acts only in the apparent absence of prior sensitization (Marshall et al., 1965). Moreover PHA normally induces mitosis in a high proportion of lymphocytes whereas only a small proportion of lymphocytes from a sensitized individual will respond to the sensitizing antigen. Consequently PHA is considered by some to be a non-specific stimulant (Grasbeck et al., 1964).

That the in vitro activity of the antigenically-stimulated lymphocytes parallels its behavior in vivo was shown in a number of studies. Gowans (1962), who used H^3-thymidine labelled lymphocytes derived from an inbred hooded strain of rats, found labelled hemocytoblasts in F_1 rats of the cross between inbred hooded and inbred albino following injection with parental thymidine-labelled lymphocytes. In this case the F_1's were genetically incapable of reacting against the parental cells. Xenogenic (i.e., between

species) stimulation produced the same result since the injection of rat lymphocytes into mice (which had been irradiated to inhibit their immune response) gave rise to proliferating cells of rat karyotype. Similarly Porter et al., (1962) injected tritium-labelled male rat lymphocytes into neonatal female rats (which were too young to respond immunologically) and observed by karyotypic analysis the proliferation of labelled male cells. In 1965 Nettesheim et al. found that a fraction of rat lymphocytes isolated in cell-impermeable chambers which were planted in mice synthesized DNA and became hemocytoblasts. The daughter cells resulting from subsequent mitosis were categorized into the plasmacyte and lymphocyte series.

The absence of an established theory of immunity limits the perspective of in vitro studies on lymphocyte proliferation when viewed with regard to the total immune response. Nevertheless since the induction of proliferation of isolated lymphocytes retains the specificity characteristic of the total immune response, their use in culture is a valuable immunological tool.

SECTION I. MIXED LEUKOCYTE REACTION AND HISTOCOMPATIBILITY

REVIEW OF LITERATURE

1. Histocompatibility and Genetics

Although the clinical transplantation of tissues to repair wounds and malformations has long been in use, up to the beginning of this century no correlation was recognized between the success of a tissue graft and the degree of relationship between the donor and the recipient (reviewed by Billingham, 1963a).

The first systematic work of transplantation involved tumor tissue in mice, the objective being the study of cancer. Although the racial character of the host was known to influence the susceptibility to a transplanted tumor (Jansen, 1902, cited in Billingham, 1963a), it was Little *et al.* (1915) who first established an adequate genetic explanation.

Gorer (1937, 1938) found that one of the genes of the mouse determined both susceptibility to a tumor and the presence of an antigen (H-2) and showed that resistance to this tumor occurring in those mice not containing the H-2 antigen was associated with high antibody levels directed against this antigen. In 1943 Gibson *et al.* pointed out the similarity between the rejection of normal skin and the development of immunological immunity. Subsequent work by Medawar (1944) and later by many others supported this contention.

It is then clear that graft rejection is genetic in origin and immunological in execution.

The strength of a histocompatibility gene (while varying with the specific allelic combinations) refers to the magnitude and rapidity of response elicited after grafting. For example the rapidity of rejection caused by an H-2 gene is about 14 days; it is about four times as long for an H-1 gene, and still longer for an H-4 gene (Snell et al., 1961). A characteristic of strong genes is their large number of functional alleles; about 20 in the H-2 of mice (reviewed by Billingham et al., 1963b) and over 30 in the B of chickens (reviewed by McDermid, 1964).

Since the standing criterion for histocompatibility is the acceptance of grafted tissues such as skin, it is evident that these tissues contain all such isoantigens. However, the distribution of isoantigens in blood cells is variable. In rabbits these are found on leukocytes but not on erythrocytes (Medawar, 1946). In mice the isoantigen H-2 is present on both cell types but erythrocytes do not carry Y, H-1, H-3 or H-4 (reviewed by Billingham et al., 1963). The strong B-isoantigen of chickens is found on both cell types (Schierman et al., 1961).

2. Methods of Studying Histocompatibility

(i) General review

A brief survey of methods of studying histocompatibility illustrates the diversity of approaches to this problem. The original and still widely used procedure of studying histocompatibility involves tissue grafting between individuals of controlled genotype. The survival of the graft is of course dependent on the immunological discretion of the host.

The reverse situation, in which immunologically competent grafted cells react against an immunologically defenseless host (such as an embryo), was used by Simonsen in 1957. This graft versus host (GVH) reaction leads to a variety of host symptoms, splenomegaly being one of the most consistent (Simonsen, 1962). The accessibility of the chick embryo has led to widespread use of the GVH reaction in studies of histocompatibility in this species (Jaffe et al., 1962; Ruth et al., 1965). Of the types of cells injected (usually whole blood), Terasaki (1959) has shown that the lymphocyte is largely or exclusively responsible for the reaction, although the resulting splenomegaly is due to proliferation of both host and donor cells (Owen et al., 1965).

Agglutination is routinely used for those histocompatibility isoantigens which are carried on erythrocytes. Specific leukocyte antigens in humans have been demonstrated but no association with histocompatibility has been made (van Rood et al., 1965, pp. 21-37). Cytotoxicity techniques are also potentially useful (Terasaki et al., 1965, pp. 83-95). Recently a method employing inhibition of sensitized

peritoneal cell migration in the presence of the antigen has been found effective in detecting a state of immunity involving histocompatibility isoantigens in mice (Al-Askari et al., 1965, pp. 17-19).

Another method, which was used in this study, will be reviewed in detail.

(ii) Mixed leukocyte stimulation

In 1961 Schrek et al. made the incidental observation that a few large nucleated cells (hemocytoblasts) were developed when leukocytes from two individuals were mixed and cultured.

Motivated by this report, Bain et al. (1964) suggested that the development of these was a result of stimulation by foreign antigens, possibly those concerned with histocompatibility. To support this suggestion they emphasized that no stimulation occurred between blood cells of identical genotypes (from monozygotic twins) but generally did in similar cultures derived from dizygotic twins. The "donor" stimulus was associated with leukocytes; erythrocytes, platelets and plasma were ineffective. Later that year, Bain et al. (1964) strengthened this interpretation by showing that sibling pairs had a lower average stimulation than either member with an unrelated individual.

In 1965 Moynihan et al., using graft exchange between 13 pairs of squirrel monkeys, showed that a modification of Bain's method had a high predictive value ($r = .8796$) for subsequent graft rejection. They showed also that the post-graft rejection response in vitro was greater than the pre-graft response.

Chapman et al. (1965), using non-inbred rabbits, showed that a similar stimulation of cell division (as measured by DNA synthesis) occurred on mixing cell suspensions derived from spleen or from lymph nodes. The response paralleled that obtained from a known secondary response in the same system.

Dutton (1965) used this method with inbred strains of rats and mice. In all of the 21 possible combinations between 7 inbred strains of mice there was stimulation in the cell mixtures whereas with isologous mixtures there was none. This coincides with known histocompatibility responses of such combinations. This result was further supported by using inbred rats and finding that the response between the F_1 and either parental strain was less than that obtained with the parent-parent mixtures.

Recently Kasakura et al. (1965) and Gordon et al. (1965) showed that the factor(s) which stimulate homologous cells in human leukocyte cultures are released into and can be recovered from the culture medium. This was anticipated by the work of Mannick et al. (1964) who showed that the cell-free fluid from a culture of rabbit spleen would specifically sensitize a recipient rabbit against the spleen cell donor.

CHICKEN LEUKOCYTE CULTURES

Review

Beginning with Carrel *et al.* (1922) cultures of chicken leukocytes have been intensively studied (see review by Jacoby, 1965). In all cases the macrophages which develop from the monocytes ultimately dominated the culture and the lymphocytes disappeared. The first proliferation of the latter cell type was achieved by Newcomer *et al.* (1963) using PHA as a stimulus.

1. Materials and Methods

(i) Criteria for analysis

Differential counts of cell suspensions used for culturing were carried out according to the descriptions of Lucas *et al.* (1961). The analysis of cultured cells presented no special difficulties. Granulocytes and thrombocytes quickly degenerated and were not considered. The lymphoid and monocyte series were easily distinguished, the latter invariably having an extensive vacuolated cytoplasm. Immature cells of the lymphoid series (i.e. lymphoblasts and immature lymphocytes) have no definitive qualitative differences from mature cells; however, they differ markedly in size. After prolonged culturing all the cells were greatly reduced in size; this was probably due to an increase in the osmotic pressure of the medium associated with cell autolysis. In both stained smears and wet mounts a lymphocyte was classified as immature when its mean diameter (i.e. the average of two

measurements taken at right angles to one another) exceeded six microns. Clearly identifiable mitotic cells had a diameter of about eight microns.

A comment should be made on terminology. Undifferentiated mammalian cells which give rise ultimately to lymphocytes are morphologically indistinguishable from those forming other cell types, hence the general term hemocytoblast can be applied. However, similar undifferentiated avian cells which give rise to lymphocytes are distinguishable, hence the specific term lymphoblast (Lucas et al., 1961).

(ii) Preparation of chicken leukocyte suspension

Except for the modifications that are mentioned later, chicken leukocyte suspensions were prepared according to the following procedure. Sterile syringes (10 or 20 ml capacity) were fitted with 20 gauge x 1-1/2" (for hens) or 18 x 1-1/2" (for roosters) sterile disposable needles (Jinton Clinical Thermometer Co.). Heparin, the anticoagulant, was used in two preparations: (i) 1000 iu/cc in saline containing benzyl alcohol as a preservative (Eli Lilly Co., Toronto) and (ii) 2000 iu/cc (Connaught Laboratories, Toronto) dissolved in preservative-free Eagles minimal medium. The former was used to "wet" a syringe, the latter was added in measured amounts depending on the amount of blood desired (0.20 for 10 cc, 0.30 for 15 cc).

Blood was drawn from the Wescodyne-disinfected wing of a 24-hour starved chicken. Contamination seldom occurred as revealed by routine plating of aliquots of blood. The contents of each

syringe were transferred to a 20 ml centrifuge tube and spun at 12 - 15 x g for 1 - 1-1/2 hours at room temperature. The supernatant contained mainly small lymphocytes and unclumped thromobocytes whereas the "buffy coat" comprised monocytes, granulocytes, clumped thrombocytes and large lymphocytes. The desired portions were drawn off in a Pasteur pipette.

(iii) pH estimation

Colorimetric pH standards were made using phenol red in a KH_2PO_4 - Na_2HPO_4 buffer adjusted to appropriate electrically measured pH's. These were in 0.2 pH unit intervals from 6.6 to 8.2. Intermediate pH's were estimated by interpolation. Culture pH's were estimated to the nearest 0.1 pH by visual comparison with these standards and by comparison among themselves by position in rank according to pH.

2. Lymphocyte Stimulation by PHA

(i) Methods and results

In order to determine the adequacy of culture methods preparatory for mixed leukocyte stimulation experiments, PHA was used as a mitotic inducer. This series consists of three experiments.

Experiment 1

This was an attempt to duplicate Newcomer's results (1963). Five cc of leukocyte suspension in plasma was obtained from chicken #258. This was diluted to 40 cc with Hanks' medium (Difco Laboratories, Detroit) containing 50 iu of penicillin G and 50 µgm

of streptomycin/cc. Ten cc of this cell suspension was put into each of two Wheaton #6 serum bottles, giving a culture depth of 20 mm. To one of these 0.16 cc of PHA-M (Difco Laboratories) solution in Hanks' medium was added to give a final concentration of 0.27 mgm/cc of culture: to the other 0.16 cc of Hanks' medium alone was added. Both cultures were swirled briefly during the first half hour and again one hour later.

Leukocyte agglutination was more pronounced in the PHA culture but at 3 days phase microscopy showed no immature lymphoid cells.

Experiment 2

This was designed to utilize a range of concentrations of different batches of PHA in conjunction with a number of birds. The large number of cultures required a change to different culture tubes; vertically incubated Leighton tubes (Bellco Glass Co., Vineland, N.J.) containing 2 cc of cell suspension (culture depth 15 mm) were employed. Leukocyte suspensions, diluted with Hanks' medium as previously described, were separately obtained from chickens #167 and #25. A PHA mixture (1 PHA-M : 2 PHA-P) was added in a series of dilutions (7.10, 1.78, 0.45, 0.11, 0.03 and 0.01 mgm/cc) to cultures from each bird. Leukocyte agglutination occurred only at high PHA concentration but no immature lymphoid cells were seen at 3 days.

Experiment 3

This was designed to expand the scope of experiment 2. The medium and culture tubes were similar to those of experiment 2. A pooled leukocyte suspension was derived from birds 25, 167, 212 and 258 and the PHA comprised a mixture (in equal amounts) of four batches of PHA (PHA-P #470575, #474075; PHA-M #466383 (all from Difco) and PHA #K5047 (Burroughs, Wellcome & Co., London)) used at two concentrations, namely, the concentration originally used by Newcomer (as in Experiment 1), and 1/3 of this concentration.

Agglutination of leukocytes was especially pronounced at the higher PHA concentration. Wright's stained smears of cultures at both concentrations at three days showed occasional lymphoblasts.

(ii) Discussion

Since the first experiment of this series was very similar to Newcomer's (1963) it was felt that its failure was due to the particular unresponsiveness of the chicken and/or the inadequacy of the particular batch of PHA. Both of these reasons are advanced for the occasional failure which occurs with a normally responsive species such as humans. The failure of the second experiment and the marginal success of the third (which might have been due to PHA and/or isoantigenic stimulation) do not clarify the situation.

Others (Hardin, 1965, personal communication; Newcomer, 1965, personal communication) have also experienced irregularity of success using PHA with chickens.

However, since 25 percent chicken plasma with 75 percent Hanks' medium (Newcomer et al., 1963) or 75 percent Morgan's 199 (Hardin, 1965, personal communcation) will support extensive lymphocyte proliferation, and since the general viability was good in the cultures of the present series, it was concluded that failure was related to the PHA-lymphocyte interaction rather than to inadequacies of the culture method. Similar culture methods were therefore used in the next series of experiments.

3. Mixed Leukocyte Stimulation

The leukocyte suspension, obtained as previously described, was used to set up 2 cc cultures which consisted wholly of cells from either bird as control or of a 1:1 mixture for the experimental. Culture vessels, except as otherwise noted, were vertical 85 x 15 mm test-tubes fitted with silicone rubber stoppers (Bellco Glass, Vineland, N.J.). Incubation was at 37° C.

(i) Assay of proliferation

Cell proliferation was evaluated by determining the concentration of immature lymphoid cells/mm^3 of culture. It was felt that a conventional analysis (i.e. ratio analysis) would be unsatisfactory since such methods assume that cell viability is the same in experimental and control cultures. Two methods were used.

The first was a conventional Wright's stained smear of a mixture of the culture to be analyzed and an aliquot of a standard chicken erythrocyte suspension. This standard was added immediately before making the smear. Although the use of these erythrocytes

complicated the procedure, other materials not present in the culture (e.g. fungal spores, synthetic particles (Dowex 1-X10), human erythrocytes) were unsatisfactory as standards. The procedure involved:

(1) smearing the culture suspension to give the ratio of
Culture erythrocytes/Culture lymphocytes; and

(2) addition of a known quantity and concentration of lymphocyte-free erythrocyte suspension to the culture suspension and smearing the mixture to obtain the ratio of
Culture erythrocytes + added erythrocytes/Culture lymphocytes.

From these ratios the total number of erythrocytes/mm^3 can be derived. Using this value as a slide standard the concentration of immature lymphoid cells can be calculated as

$$\text{Immature lymphoid cells/mm}^3 = \text{Immature lymphoid cells counted } \left(\frac{\text{erythrocytes/mm}^3}{\text{(erythrocytes counted)}}\right)$$

For proof of this see Appendix 1.

The second method was a direct phase contrast count of immature lymphoid cells in a Petroff-Hauser bacteria counter. The four corner squares were counted per sample; the volume assayed then being 0.08 mm^3. Usually six samples were made of each culture.

(ii) Results

Eagle's minimal media and 25 percent chicken plasma were used to set up the cultures. Blood from two birds (Nos. 215 and 110) was used:

Bird #	Geno.	Erythro.	Lympho.	Mono.	Granulo.	Thrombo.	Total
215	$B^{13}B^{13}$	354	562	125	104	935	2080/mm^3
		(17)	(27)	(6)	(5)	(45)	(100 percent)
110	$B^{2}B^{14}$	365	600	104	131	1410	2610/mm^3
		(14)	(23)	(4)	(5)	(54)	(100 percent)

After several hours of incubation the pH of 110 was 7.3, of 215 7.4 and of the 215 - 110 mixture, 7.3 - 7.4.

Analysis was started 5 days after the beginning of incubation with the reported maximum proliferation with humans. Three cultures, comprising the two controls and the mixture were analyzed by the smear method with the following results (total counted in brackets):

Culture	Lymphocytes/mm	Immature lymphoid cells/mm
215	152 (243)	1.3 (2)
215 - 110	160 (256)	15.6 (25)
110	132 (211)	3.1 (5)

The concentration of the immature lymphoid cells in the mixture is significantly higher than the average of the control. See Appendix 2.

Measurements of pH, taken at the fifth day for the three cultures (each in five replicates), were as follows:

	110	215 - 110	215
	7.1*	7.0*	7.4*
	7.1	7.2	7.4
	7.2	6.8	7.4
	7.2	7.2	7.4
	7.2	7.0	7.3
$\bar{x}$	7.16	7.04	7.38

* Those cultures analyzed for cell morphology.

Analysis of variance of pH shows a significantly lower pH in the experimental series as compared to the mean of the controls. See Appendix 3.

An association between immature lymphoid cells and macrophages was frequently seen in the smears of the mixed culture. Since all other cell types were widely dispersed, this was interpreted as a specific interaction. Further analysis was impossible since by day 6 all the cultures had degenerated. A similar experiment is described in Appendix 4.

Repeated attempts to confirm the results of these experiments were unsuccessful. In all cases the cultures had degenerated after four or five days of growth and no cell count was possible. The condition of the experimental cultures parallelled that of the most rapidly degenerating of the controls; measurements of pH followed a similar trend.

Efforts were then devoted to changing some of the variables associated with mutual lymphocyte stimulation, as well as to culture variable affecting lymphocyte viability.

(iii) Factors affecting chicken leukocyte viability

A number of variations in the culturing procedure had no noticeable effect on the viability of lymphocytes. These included:

(a) Heparin, with and without the associated preservative, benzyl alcohol;

(b) Method of cleaning glassware, especially culture tubes. Micro-solv, (although used routinely afterwards) as compared to sulfuric acid chromate;

(c) Type of glass, new Kimax, new Pyrex and used tubes of the same brand;

(d) pH's of about 7.3, 7.4 and 7.6; and

(e) Cell concentrations of about 2,000, 4,000 and 8.000/mm^3.

It was noted that the macrophages proliferated rapidly in the early stage of culturing but with time they became vacuolated. The vacuoles finally obscured the nucleus, and at this stage all the cell types rapidly degenerated. Some of these cells had a specific gravity less than that of the culture medium; they could be seen as a ring on the culture tube at the air-liquid interface and they did not sink in the hemocytometer chamber. The vacuolar contents were considered to be lipoidal because of their high refractility and the reduced specific gravity of the cell.

Acting on a suggestion by Dr. Morgan, lower plasma concentrations in conjunction with more complete synthetic media were tried. Accordingly a new medium, NCTC 109 (Baltimore Biological Associates) was used in combination with four different levels of plasma: 0, 5, 13 and 27 percent.

Macrophages in 0 percent plasma had few vacuoles and did not appear to proliferate; lymphocytes appeared morphologically normal. Macrophages in the presence of plasma of all concentrations consistently developed large vacuoles; the proliferation of these cells appeared to increase as the plasma percentage increased. It was then evident that plasma was closely associated with the condition of macrophages in these cultures.

It is known that the lipid content of the plasma of laying hens (used in this investigation) is higher than it is for males and for non-laying females (Sturkie, 1954, pp. 28, 29). Consequently a trial was run in which leukocytes from a laying hen were washed free from autologous plasma and cultured in NCTC 109 with 25 percent autologous plasma in one case and with the same concentration of rooster plasma in the other. The cultures containing rooster plasma supported a rapidly-proliferating population of macrophages, and these contained no large vacuoles whereas those in autologous plasma developed the typical vacuolated condition.

At this point an experiment utilizing mixed leukocyte stimulation was set up using the same birds and materials as for the previously described experiment except that the culture media was NCTC 109 and 15 percent rooster plasma. By three days the pH

of all the cultures had decreased markedly and cytological analyses showed only the occasional lymphocyte remaining in a massive population of macrophages. At this point an impasse was reached. Clearly the combination of variables which led to the results of the experiment described in detail had not been elucidated.

Nevertheless, it was important to be sure that at least under some conditions the mixed leukocyte reaction would occur with chickens. The cytological data, while significant, were not adequate to assess experimental error but the pH information was both significant and complete. The observed pH change, while potentially useful *per se* for this technique, would provide convincing support only if it could be correlated with a response shown to be positive by cell proliferation. It was evident that if the pH change was associated with the increased metabolic activity resulting from mixed leukocyte stimulation, then it should be characteristic of similar experiments with human cells. After outlining the method used, such an experiment will be described.

HUMAN LEUKOCYTE CULTURE

1. Materials and Methods

(i) Preparation of cultures

Blood was drawn from an arm vein of male donors, who had not eaten for at least 6 hours, into a 10 ml vacuum tube (Type 3200 Becton, Dickinson & Co.) to which 0.30 of 1,000 iu/cc of preservative-free heparin in Morgan's 199 medium had been previously added.

The blood from each tube (9 - 9.5 cc) was transferred to a 15 ml centrifuge tube and spun at 150 g for 35 minutes at room temperature. The supernatant plasma and buffy coat (representing about 45 percent of the total blood volume) was transferred with a Pasteur pipette to a clean centrifuge tube. After sedimentation at room temperature for 3/4 to 1-1/2 hours the supernatant was withdrawn as completely as possible without disturbing the buffy coat.

The culture medium was 80 percent 199 medium (Baltimore Biological Laboratories) containing 50 iu/cc of penicillin, 50 μgm/cc of streptomycin and 20 percent autologous plasma. Each culture consisted of 1 ml of cell suspension (2 - 4000 cells/mm) in a vertical 85 x 15 mm Kimax test tube fitted with a silicone rubber stopper. Incubation was at 37° C.

An experimental method requiring a small amount of material per culture was considered important since in higher organisms, and particularly in humans, an adequate number of cultures of identical genotype can normally be derived only from a single donor. This is especially important for the mixed leukocyte reaction. Positive results were obtained from cultures containing less than 1/4 the number of cells of those previously reported.

(ii) Assay of cell proliferation

Cell proliferation was assayed by total-culture DNA synthesis as shown by incorporation of tritiated thymidine. After varying periods of growth 0.35 ml of a solution of tritiated

thymidine (1.9 μc/mm; Schwarz Bioresearch, Inc., diluted with 199 medium) containing 3.86 μc/ml was added to each culture to give 1 μc/cc of culture. Incubation was continued for 12 hours at which time the cells were scraped free of the culture tube and transferred to a 15 ml centrifuge tube. The culture tube was rinsed with 1 ml of sterile saline and added to the original suspension.

Two different methods of separating the cells from the unincorporated thymidine were compared. Chapman et al. (1965) used a perchloric acid-methanol procedure and found that 95 percent of the resulting activity was incorporated into DNA; Bain et al.(1964) used a simpler method. A comparison between these methods gave similar results and, although the preparation by Chapman's method was more stable in the scintillating solution (2.2 percent loss as compared to 5.7 percent in the first 12 hours), Bain's method was used throughout because of its greater simplicity. Bain's method will therefore be described.

The cell suspension and rinse (total, 2.35 ml) was spun for 20 minutes at 150xg and 17 - 19° C in a refrigerated centrifuge (International Equipment Co., Boston). The supernatant was taken off until 0.2 ml was left, to which 2.0 ml of sterile 0.88 percent saline was added and the cells gently resuspended with a siliconized Pasteur pipette. The next centrifugation was for 15 minutes at 100xg and the supertatant was taken off to 0.1 ml and again 2.0 ml saline were added. The latter procedure was repeated three times, the last saline wash was removed as completely as possible. The cells were then hydrolyzed. A recent procedure described by Petroff et al.

(1965) was tried, but because of inconsistent counting efficiencies it was discarded in favor of Bain's (1964) procedure. This procedure involves the addition of 0.5 ml Hyamine 10X (Packard Instrument Co., Illinois) to the sedimented cells in each centrifuge tube and storing these in the dark at room temperature for several days. At this time pure methanol was added to make up for any losses by evaporation and the contents of each tube were transferred to a counting vial (Nuclear Chicago Corp.) together with two 0.3 ml rinses of the centrifuge tube with 98 percent ethanol. Hydrolysis was completed by incubating at 75° C for 1-1/2 hours.

To the cooled vial, 19.5 ml of scintillation solution was added. This solution consisted of 0.3 percent 2,5 diphenyloxazole (DPO) and 0.01 percent 1,4-di- [2-(phenyloxazolyl)] (POPOP) (both obtained from Kent Chemicals Ltd., Vancouver) in Fisher Reagent Grade toluene.

Radioactivity was determined using a Packard Model 3003 liquid scintillation counter after the vials had been dark-adapted and cooled for one hour (to 12° C) in the storage chamber. The window used was 50 - 1000 through which the tritium standard was counted with an efficiency of about 49 percent.

The total disintegrations per minute (dpm) for each sample was not determined since the counts per minute (cpm) on samples of consistent counting efficiency was considered adequate. The counting efficiency of each sample was determined through the 50 - 1000 window by subtracting the sample count from that of the external standard. The average deviation from the mean of a typical experiment was 0.27 percent of the mean.

2. Mixed Human Leukocyte Stimulation

The experiment was designed as a randomized complete block. From two unrelated male donors, cell suspensions of the following compositions were used to set up mixed and control cultures:

Donor	Erythro.	Lympho.	Mono.	Granulo.	Total
KTP	1472	582	35	789	2878/mm^3
	51.2	20.2	1.2	27.4	100%
WTH	1833	433	16	455	2737/mm^3
	66.9	15.8	0.6	16.7	100%

After several hours of incubation the pH's of KTP cultures was 7.1, of WTH 7.2 and the mixture was intermediate i.e. 7.1 to 7.2.

Four replicates were harvested after 120 hours of growth, and the following results (cpm x 10^2; pH in brackets) were obtained:

KTP	KTP - WTH	WTH
66 (7.1)	45 (7.0)	75 (7.2)
2 (7.1)	30 (7.1)	2 (7.2)
2 (7.1)	128 (7.0)	7 (7.2)
4 (7.1)	79 (7.1)	24 (7.2)

There is a trend to higher DNA synthesis ($p < 0.1$) and a significantly lower pH ($p < .05$) in the mixture as compared to the average of the controls (see Appendices 4 and 5).

After 148 hours the three remaining replicates were harvested with the following results:

KTP	KTP - WTH	WTH
29 (7.1)	122 (7.1)	2 (7.2)
50 (7.1)	159 (7.0)	3 (7.2)
26 (7.1)	179 (7.0)	84 (7.2)

The same trend is present and more pronounced than in the previous results. See Appendices 6 and 7.

Despite the variability it is possible to generalize that in the mixed cultures there is a significantly greater rate of cell proliferation and a concomitantly lower pH.

In considering this association as functional (with reference also to chickens and omitting the experiment involving excessive macrophage proliferation) the following points should be made:

(1) In experiments in which the cells degenerated (the other experiments were successful) the pH values of the mixed cultures were never below the lowest control value and were generally as high as the highest control.

(2) In the experiments which were successful a pH decrease in the mixed cultures was observed only after prolonged incubation (more than 3 days).

DISCUSSION

The effect of laying hen plasma on macrophages appears not to have been noted previously. Although standard methods of preparing chicken plasma and sera generally specify the use of males, the reasons advanced concern their greater hardiness and greater blood quantity. It is of interest that chick-embryo extract induces a similar morphological change in chicken macrophages (Jacoby, 1964, p. 57). Since the lipid in the blood of the laying hen is deposited in the egg (Romanoff, 1949, p. 241), this association is not surprising.

The association noted between pH change and DNA synthesis in mixed human leukocyte cultures support the contention that in similar cultures of chicken cells these variables are similarly related. The justification for disregarding the experiments which failed rests on the conclusion that failure was due to technical causes, viz. inadequate culture conditions and overgrowth of lymphocytes by macrophages.

Unfortunately the repeatability of mixed leukocyte stimulation in chicken cultures was so poor that the original hypothesis, relating the <u>in vitro</u> reaction with incompatibility at the B locus, could not be fully tested. The proof of this would be shown by:

(1) mixed leukocyte stimulation between incompatible B genotypes, and

(2) an absence (or reduction) of such stimulation with identical B genotypes.

The first criterion was met, the second could not be tested.

For theoretical and technical reasons the role of macrophages in mixed leukocyte cultures must be assessed. Pertinent to this is the uncertainty of whether the observed stimulation represents a primary or a secondary response.

According to a recent theory of immunity the first stage of the response involves the macrophage which, upon phagocytosis and recognition of an antigen as being foreign, transfers information (presumably an RNA-antigen complex) to a previously-insensitive population of lymphocytes which then responds specifically against this antigen (Fishman, 1961; Fishman et al., 1963).

Such a physical association (suggesting such a transfer) between macrophages and lymphocytes (which subsequently became hemocytoblasts) has been observed in mixed human leukocyte cultures (MacFarland et al., 1965). The most obvious interpretation is that at least in mixed leukocyte cultures of human origin the response is primary and that macrophages are indispensible. On the other hand, it is possible that this association represents sensitized lymphocytes attacking foreign macrophages.

However, the former assumption was extended to chicken cells, and suspensions with a relatively high monocyte count (i.e., up to 5 percent) were selected for culturing. The likelihood of this assumption was increased by noting in the experiment described an apparent association between immature lymphocytes and macrophages. Even so, unless macrophage proliferation is curbed by culture components, such as laying hen plasma, lymphocytes have little chance of survival beyond a few days.

Nevertheless, doubt can be cast on the previous interpretation of macrophage function in immunity and/or its necessity for a positive response in mixed leukocyte cultures. Terasaki (1959) showed that a population consisting of 97 - 98 percent lymphocytes, obtained from non-immunized donors, would cause splenomegaly in chick embryos. If one assumes: (1) that the contaminating cells were not monocytes, and (2) that lymphocytes do not transform into monocytes, then either the chicken lymphocytes were already sensitized to histocompatibility isoantigens and/or these cells were, by themselves, competent to initiate a primary response.

Related to the first assumption is that a similar competence of an even more pure preparation of lymphocytes in other species will similarly elicit a GVH reaction (Gowans, 1962). However, it must be remembered that in such differentials the absolute exclusion of another cell type is virtually impossible. Evidence for such prior sensitization is the cross reactivity observed between histocompatibility isoantigens in guinea pigs and antigens of a number of bacterial genera (Chase et al., 1965; Rapaport et al., 1965).

The possibility of lymphocyte transition to monocytes also cannot be excluded. With human cells such attempts have given conflicting results (Rabinowitz, 1964; Gough et al., 1965). Clearly this type of work rests on the virtually untenable assumption previously mentioned. Carrel et al., (1922) suggested that in cultures of chicken cells the macrophages arose from monocytes and my observations concur since in the occasional cultures (not used for mixed lymphocyte stimulation), in which no monocytes were noted in the differential

count made prior to culturing, the macrophages - when they were seen - represented a rapidly-proliferating population arising late in culture life. If they did not arise from the occasional monocyte which had been missed in the differential then at least the transition of a lymphocyte to a monocyte (and thence to a macrophage) is indeed a rare event under culture conditions.

If macrophages are not essential for a mixed leukocyte reaction, then selection against monocytes should result in improved culture success. This has not been tested.

The proliferation in mixed leukocyte cultures then depends on, among other things:

(1) The role of the primary response, and if it is responsible the optimal proportion of cell types, if more than one, involved. Even in the absence of active participation in immunity, phagocytic cell types would compete with lymphocytes for isoantigen. If primary response is largely or completely absent, then opportunity for exposure to cross-reacting antigens will be another variable.

(2) The complex interactions affecting the proliferation of cells which are the target as well as the aggressor in the immune response.

Obviously the mixed leukocyte reaction is only useful for gross comparisons in isoantigenic studies. Fundamental information on isoantigenic stimulants depends not only on the separation of these stimulants from living cells but also from each other. The ultimate significance of leukocyte cultures in this regard will likely be as a bioassay in this task.

SUMMARY

The initial objective of the study was to relate the mixed leukocyte reaction to specific genetic loci. The positive stimulation obtained in early experiments was not repeatable. Attempts to identify the cause of failure revealed that the high lipid content of the plasma was responsible for culture degeneration. However, the use of plasma containing less lipid promoted macrophage proliferation to such an extent that the lymphocytes were destroyed within a few days. It was noted that a lowering of the pH was a constant feature of the mixed cultures in those experiments considered successful by cytological analysis.

In order to confirm the cytological data obtained with chickens it seemed worthwhile to investigate further the observed pH changes by using mixed human leukocyte culture and measuring cell proliferation by incorporation of tritiated thymidine. It was shown with these cells that a positive response in the mixed cultures was associated with a significantly lower pH.

It was unfortunately not possible to identify the factors which prevented success in relating enhanced mixed leukocyte proliferation to histocompatibility antigens. However, the data obtained were not inconsistent with the hypothesis that the relationship exists. In the course of the experiments a method was developed for evaluating the concentration of immature lymphoid cells in the *in vitro* culture.

SECTION II. HORMONAL EFFECTS ON HUMAN LYMPHOCYTE PROLIFERATION

The effect of hormones on immunity has been the subject of much investigation. However, in addition to the variability of results from animal experimentation and the uncertainties inherent in extrapolating these to humans, such *in vivo* information gives no indication of the direct effect on a specific cell type, knowledge of which is important for the experimental use of cells in culture and also for information on the *in vivo* mode of action of the hormone.

In 1963 Mauer published a brief report in which he related karyotypic alterations of lymphocytes *in vitro* to the presence or absence of specific hormones. He found that PHA-stimulated lymphocytes displayed not only karyotypic alterations but also, in some cases, a change in mitotic index when specific hormones were added.

It was in the hope of confirming and extending Mauer's observations that this study was undertaken. In a preliminary experiment a mixture of hormones was added to an *in vitro* culture of PHA stimulated lymphocytes and an alteration in cell proliferation was observed. Encouraged by this result, the hormones of the mixture and others were then used individually in the experiments which follow.

MATERIALS AND METHODS

Human male leukocyte cultures were set up as described for "controls" in the previous section except that PHA was added. A comparatively low level of stimulation was used in these studies because:

(1) culture conditions were less subject to changes induced by cell metabolism; and

(2) a large prcportion of lymphocytes would be only moderately stimulated, making it likely that factors which were potentially capable of affecting lymphocyte proliferation would have optimal opportunity to act.

In all the experiments to be described the stimulant was 14 μgm/cc of PHA, representing 1/5 that recommended by the manufacturer (Burroughs, Wellcome & Co., London).

The preparation of steroid hormones for addition was as follows: the hormone was dissolved and also sterilized in absolute ethanol and added to a sterile 100 ml bottle. The control consisted of a similar amount of alcohol in a second bottle. These containers, with loosened tops, were subjected to high vacuum for 2-1/2 hours at which time all the liquid had evaporated. Culture media was then added and the hormone redissolved by agitation for 3 hours at 37° C. At this stage the hormone concentration was four times that of the final culture.

In order to expose the cells simultaneously to PHA and the hormone being tested, the latter were mixed together before addition to the previously dispensed cell suspension.

The comparison between the experimental and control series generally started after 36 hours of culture and was based on three consecutive 12 hour periods of DNA synthesis. Twelve hours was considered to be the upper limit of exposure to tritiated thymidine. Initially the 24 - 36 hour period was also measured but the low level of DNA synthesis at this time did not warrant the extra materials required. Each experiment used 24 to 36 cultures.

The experimental designs were of two types, completely randomized and randomized complete block. In the latter case each block was derived from the same cell dispensing pipetteful (which contained enough for six cultures).

HUMAN CHORIONIC GONADOTROPIN

Human chorionic gonadotropin (HGC) is a glycoprotein of molecular weight about 30,000 (see review by Geschwind, 1963, p. 16). The maternal plasma concentration reaches a peak of 100 - 120 iu/cc in the first trimester of pregnancy in humans and declines thereafter to 5 - 35 iu/cc (see review by Rowlands, 1963, pp. 96, 97).

No *in vivo* studies have been done solely on the effect of HCG immunity. The sole *in vitro* study was that of Mauer (1963) in which doses "ten times the usual circulating concentration in pregnant women" were found to reduce lymphocyte proliferation.

Because of its wide variation during the course of pregnancy such a description of concentration is not very informative. In the experiments to be described an arbitrary value of 30 iu/cc was used. Two experiments were carried out, using HCG from different sources (lot #54387, Calbiochem, Los Angeles, and lot #P1905 (activity 3570 iu/mgm) Mann Research Laboratories, New York).

Since the results were similar, only the experiment using HCG from the latter source will be described.

The cultured cell count was as follows:

Erythro.	Lympho.	Mono.	Granulo.	
2059	510	46	265	2880/mm^3
71.5	17.7	1.6	9.2	100%

The design was randomized complete block.

There is a significant decrease of proliferation in the presence of this hormone. See Figure 1 and Appendix 8. This effect is significant at 36 - 48, 48 - 60, and 60 - 72 hours of culture. In the experiment not described 300 iu/cc was lethal at 24 hours.

A critical factor in this experiment is the purity of the HCG preparation. Although the products used were of the highest available purity there is some doubt that pure HGC has ever been prepared (Loraine, 1961, p.287). The presence of an active contaminant is suggested by the observed cell death in the presence of high (300 iu/cc) HCG concentration since values exceeding this have been found in pregnancy, although no concomitant observations on immunity or lymphoid tissue were reported (see review by Rowlands, 1963, p. 97).

On the other hand, prolonged exposure of lymphocytes to to high levels of this hormone may not occur <u>in vivo</u> since lymphocytes move intermittently to the extravascular tissues, where concentrations of HCG are lower (Bruner, 1951).

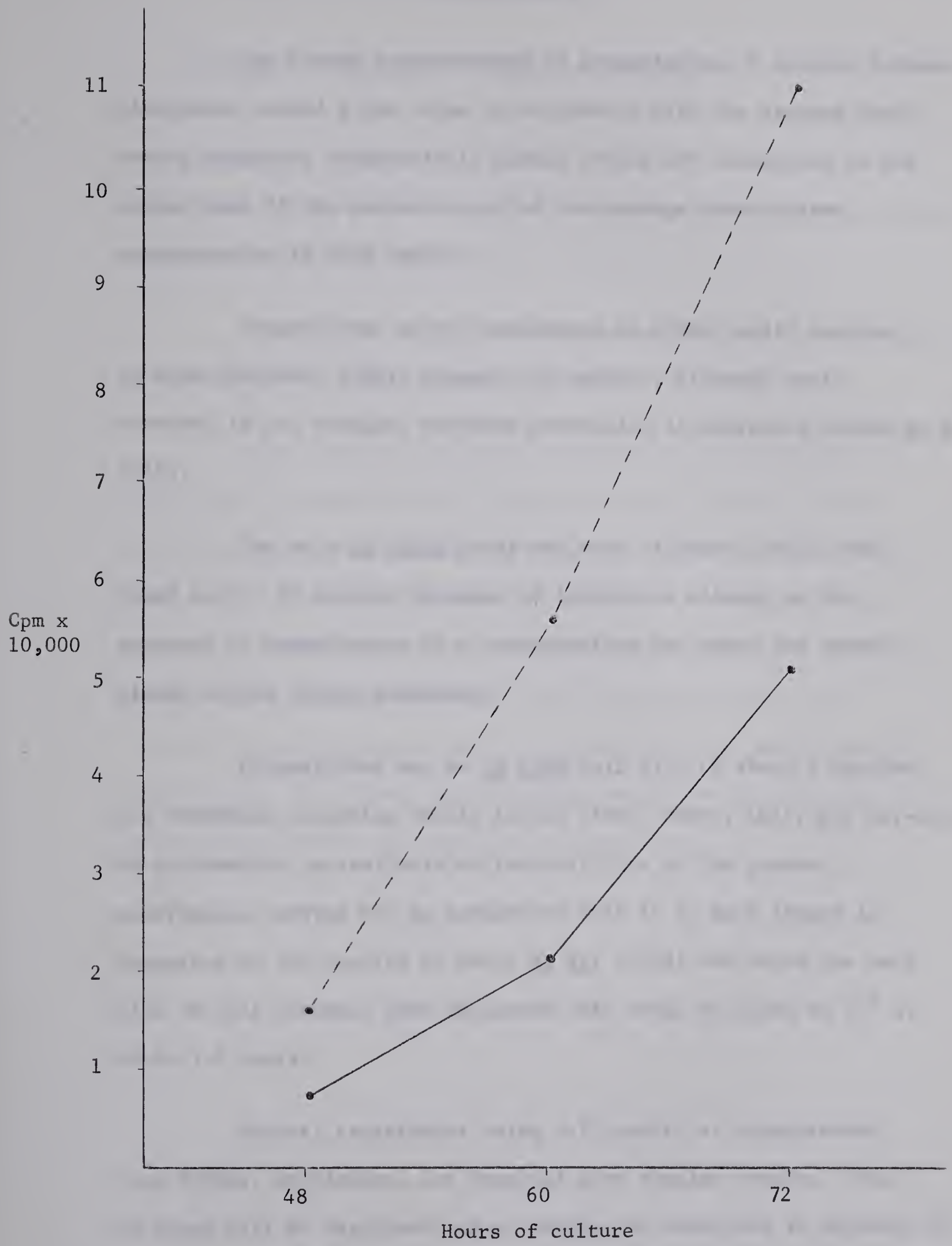


Fig. 1. Effect of HCG (30 iu/cc) on DNA synthesis. Each point is the mean of 6 cultures for a 12 hour period added to the mean of the preceding periods (starting at hour 36) to give a cumulative measure of DNA synthesis.
(HCG ——; control ----)

PROGESTERONE

The plasma concentration of progesterone, a steroid hormone, fluctuates around a low value in accordance with the estrous cycle. During pregnancy progressively higher levels are found, and in the latter half of the gestation period the average human plasma concentration is 0.10 μgm/cc.

Progesterone is not considered to affect graft survival in mice (Medawar, 1956); however, in rabbits, although graft survival is not changed, antibody production is decreased (Hulka *et al.*, 1965).

The only *in vitro* study was that of Mauer (1963), who found a 20 - 50 percent decrease of lymphocyte mitosis in the presence of progesterone at a concentration ten times the normal plasma levels during pregnancy.

Progesterone has an *in vivo* half life of about 5 minutes, the breakdown occurring mainly in the liver (Short, 1961, pp. 427-431). No information is available on the half life in the present experimental system but an indication that it is much longer is suggested by the results of Short *et al.* (1958) who found the half life of this hormone, when incubated with whole ox blood at 37° C, to be 7.2 hours.

Several experiments using 0.10 μgm/cc of progesterone (lot #53322, CalBiochem, Los Angeles) gave similar results. One of these will be described below, another is described in Appendix 12.

The cell suspension consisted of:

Erythro.	Lympho.	Mono.	Granulo.	
1052	499	21	148	$1720/mm^3$
61.2	29	1.2	8.6	100%

A randomized experimental design was used, a factor which contributed to the relatively high experimental error.

There was no significant difference between the experimental and control. See Figure 2 and Appendix 9.

With respect to hormonal breakdown it is reasonable to think that if the effect of progesterone were primarily on the initial stages of cell stimulation then its effective concentration would be only slightly less than that added. If the effect were mainly at a later stage, the first measured period of DNA synthesis would be most affected (provided that progesterone were still effective at this reduced level). The data gave no indication of either, hence it appears that progesterone at normal pregnancy levels has no effect on lymphocyte proliferation.

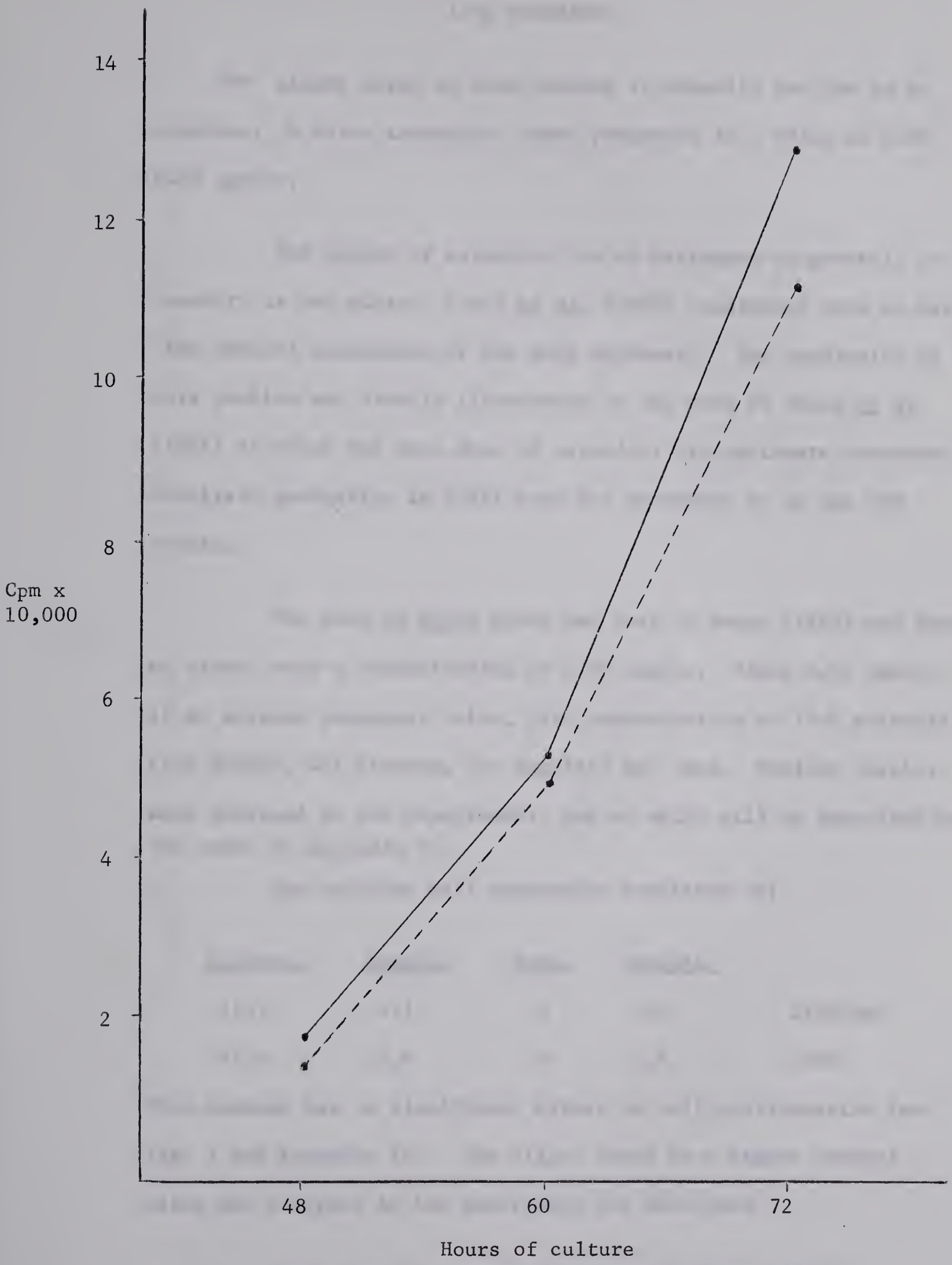
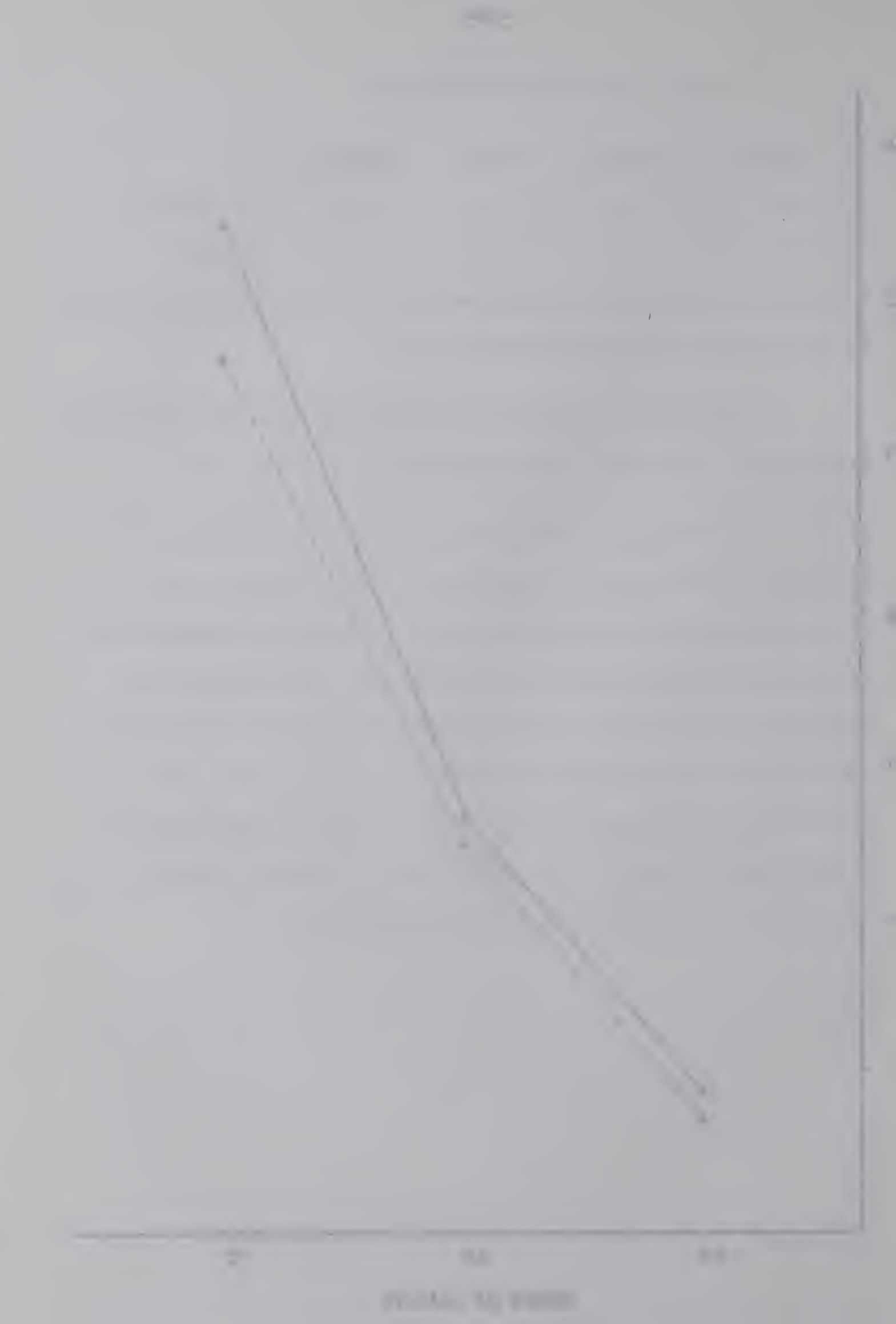


Fig. 2. Effect of progesterone (0.1 µgm/cc) on DNA synthesis. Each point is the mean of 4 cultures for a 12 hour period added to the mean of the preceding periods (starting at hour 36) to give a cumulative masure of DNA synthesis. (Progesterone ——; control ----).



17-β ESTRADIOL

The plasma level of this hormone is normally too low to be detected. It rises throughout human pregnancy to a value of 0.02 - 0.03 μgm/cc.

The effect of estradiol (or of estrogens in general) on immunity is not clear. Nicol et al. (1964) considered them as being "the natural stimulants of the body defences." The complexity of this problem was clearly illustrated by the work of Stern et al. (1955) in which the same dose of estradiol diproprionate increased hemolysin production in C57Bl mice but decreased it in the C3H strain.

The sole in vitro study was that of Mauer (1963) who found no effect with a concentration of 0.01 μgm/cc. Since 0.01 μgm/cc is an average pregnancy value, this concentration of 17-β estradiol (lot #54554, Cal Biochem, Los Angeles) was used. Similar results were obtained in two experiments, one of which will be described below, the other in Appendix 14.

The cultured cell suspension consisted of:

Erythro.	Lympho.	Mono.	Granulo.	
1727	471	0	90	2288/mm^3
75.5	20.6	0	3.9	100%

This hormone has no significant effect on cell proliferation (see Fig. 3 and Appendix 10). The slight trend to a higher control value was reversed in the experiment not described.

These results concur with those of Mauer (1963).

(Cont'd)

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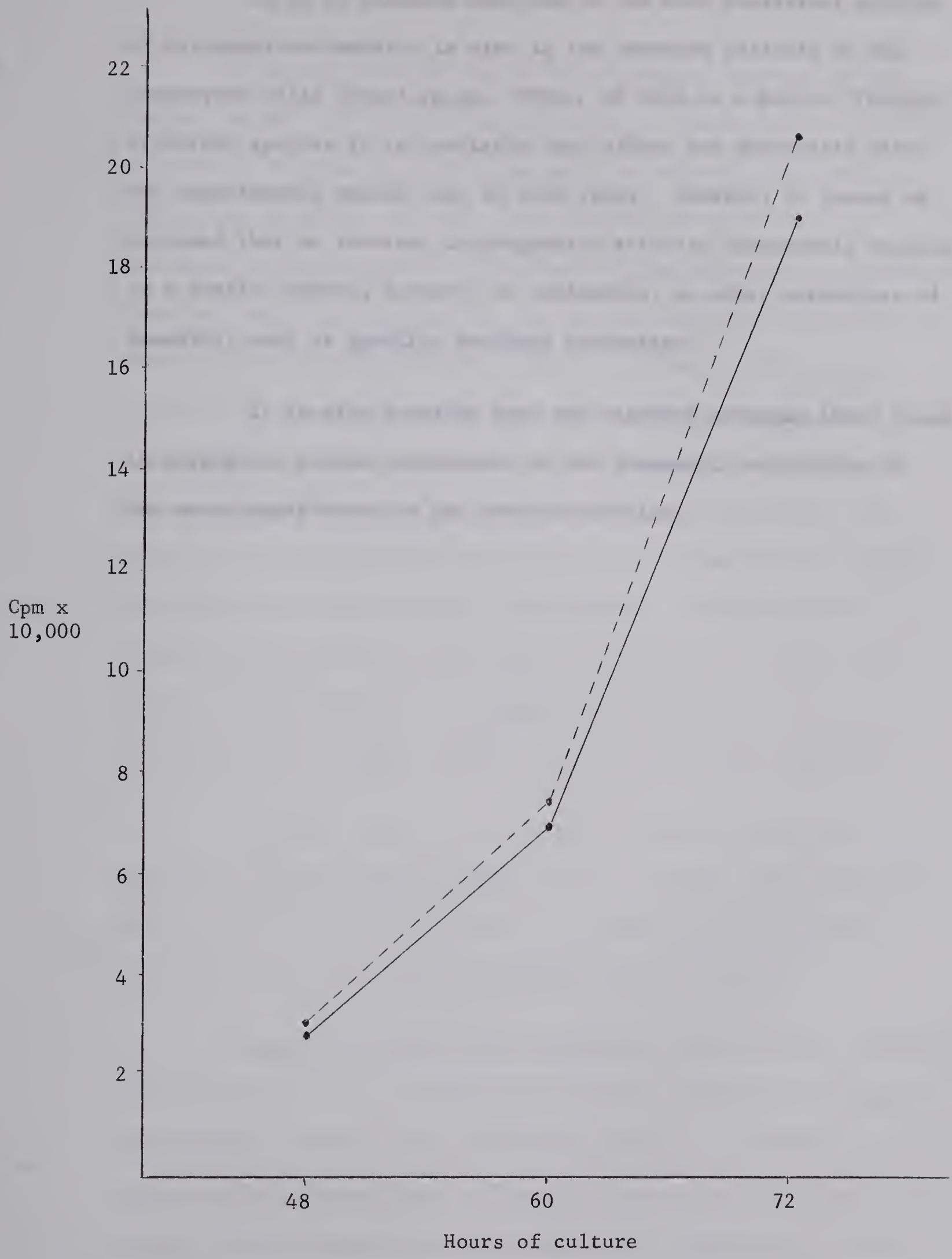
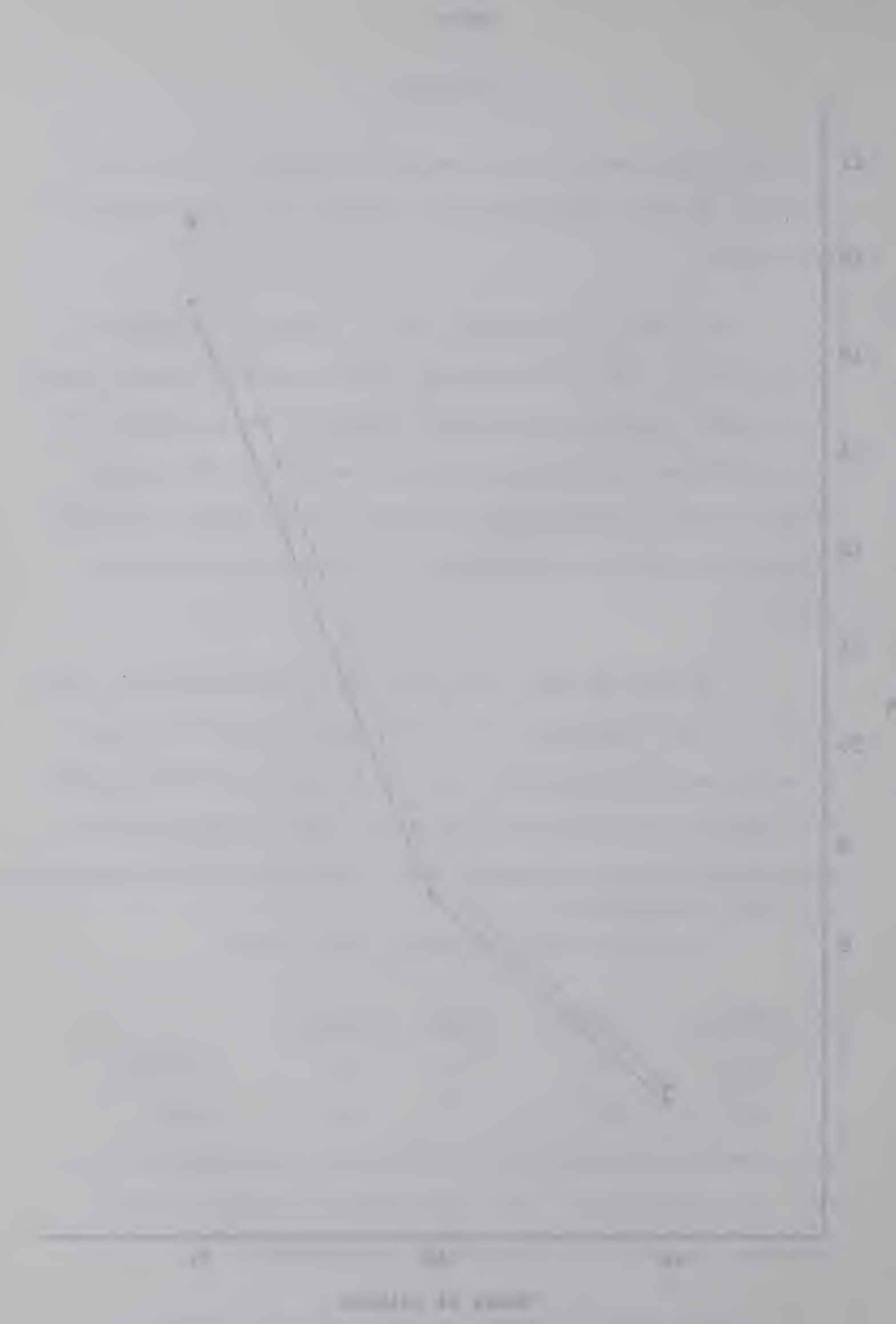


Fig. 3. Effect of 17-β-estradiol (0.01 μgm/cc) on DNA synthesis. Each point is the mean of 5 cultures for a 12 hour period added to the means of preceding 12 hour periods (starting at hour 36) to give a cumulative measure of DNA synthesis. (Estradiol ——; control ----).



It is of interest that one of the more consistent effects of estrogens on immunity in mice is the enhanced activity of the phagocytic cells (Nicol et al., 1964). If this is a general feature in different species it is precisely that effect not detectable with the experimental method used in this study. However, it cannot be supposed that an increase in phagocytic activity necessarily results in a similar effect, directly or indirectly, on other parameters of immunity, such as specific antibody production.

It is also possible that the elevated estrogen level found in laying-hen plasma contributed to the phagocytic activities of the macrophages noted in the previous section.

HYDROCORTISONE

Hydrocortisone is one of many hormones produced by the adrenal cortex. The normal concentration in plasma is 0.15 - 0.20 μgm/cc, but it rises during conditions such as pregnancy or stress to 0.50 μgm/cc or more. However, large amounts of hydrocortisone are bound by serum proteins ("transcortin" and albumin) and rendered inactive, hence the activity of the hormone depends on its absolute concentration and the extent to which it is bound. For example, although the absolute concentration rises during pregnancy the transcortin level also increases and the hormonal activity rises only slightly (Cope, 1964, ch. 2). However, during stress ("any threat to the equilibrium or metabolic homeostasis of the organism" Cope, 1964) the hydrocortisone rises quickly and, although no concomitant information is available on the level of transcortin under these circumstances, *in vivo* evidence indicates that this hydrocortisone is largely unbound (Cope, 1964, 51-55, & Ch. 2).

The only report of the effect of hydrocortisone on lymphocyte proliferation *in vitro* is that of Mauer (1963) who found that concentrations of 0.50 μgm/cc increased the mitotic index by 50 percent although 5.0 μgm/cc apparently had no effect.

Several experiments were carried out with culture additions of 0.05 and 5.0 μgm/cc of hydrocortisone (lot #54144, Cal Biochem, Los Angeles). Unlike the previous experiments the hormonal concentration of the plasma used must be considered. This was estimated as 0.15 μgm/cc of whole plasma which would contribute

0.03 μgm/cc in the culture. Hence the total culture concentrations would be 0.53 and 5.03 μgm/cc. In the experiment to be described two periods of DNA synthesis (48 - 60 and 60 - 72 hours of culture) were measured. This was necessitated because of the large number of treatments required.

The cultured cell suspension consisted of:

Erythro.	Lympho.	Mono.	Granulo.	
5143	411	40	271	5865/mm^3
88	7	0.7	4.3	100%

A randomized complete block design was used.

There was a significant difference between all treatments. See Table 1. The same trend is shown in an experiment described in Appendix 15.

There is no apparent explanation for the present disagreement with Mauer's results. Possibly it is associated with the sequence of cell exposure as noted with Prednisolone (Elves et al., 1964).

Although the lower level of hydrocortisone used in this experiment coincides with that occasionally found in vivo there is not necessarily a precise correspondence in effective concentration. However, it is evident that this hormone at an effective concentration similar to that found under some circumstances in vivo profoundly affects lymphocyte proliferation. Significant effect on lymphocyte cultures could then be expected with the use of plasma obtained from donors which were pregnant or under stress. This effect would be modified by the usual dilution of plasma for this purpose.

Table 1. Effect of hydrocortisone on DNA synthesis (cpm x 100) in each culture

Period of DNA synthesis (hrs)	48 - 60			60 - 72		
Conc. of hydrocortisone[a] µgm/cc	0	0.5	5.0	0	0.5	5.0
Block						
1	420	35	29	700	35	26
2	589	48	31	854	40	30
3	524	61	52	694	58	23
4	542	49	25	715	41	19
5	339[b]	60	19	505	42	28
6	459	44	25	669	37[b]	18
$\bar{x}$	478.8	49.5	30.2	689.5	42.2	24

ANOV

Source	s.s.	d.f.	m.s.	F	F .05
Treatment	2,401,985	2	1,200,993	23.05**	4.46
Block	34,077	5	6,815	13.08**	3.69
Period	38,875	1	38,875	74.62**	5.32
Treatment x block	64,493	10	6,449	12.38**	3.35
Treatment x period	94,542	2	47,271	90.73**	4.46
Block x period	2,734	5	547	1.05	3.69
Error	4,167	8	521		
Total	2,640,873	33			

The least significant difference (.05) (Snedecor, 1964, p. 251) is 30.4. The 0.0 level is significantly different from either 0.5 or 5.0 but the latter are not significantly different from each other. The 0.0 level was then omitted to allow a closer comparison between the 0.5 and 5.0 levels.

[a] Addition to estimated plasma contribution of 0.03 µgm/cc

[b] Estimates of missing values.

Table 1. (continued)

Comparison of effects of 0.5 and 5.0 μgm/cc of hydrocortisone on DNA synthesis

ANOV

Source	s.s.	d.f.	m.s.	F	F .05
Treatment	2109	1	2109	23.23**	7.71
Block	848	5	169.6	1.87	6.26
Period	273	1	273	3.01	7.71
Treatment x block	260	5	52	.57	
Treatment x period	3	1	3	.03	
Block x period	124	5	24.8	.27	
Error	363	4	90.8		
Total	3980	22			

Hydrocortisone seems not to interfere with the "recognition" of an antigen, hence its time of action follows this stage. This is apparent from the work of Roseneau et al. (1962) who, using an in vitro system in which mouse lymphocytes sensitized against strain-L cells destroyed the latter in vitro, found that in the presence of 25 - 150 μgm/cc of hydrocortisone the lymphocytes attached to the target cells (i.e. "recognized" them) but were unable to destroy them. The viability of either cell type (judged by morphological criteria) was unimpaired even at this concentration of hormone.

The relatively small reduction in DNA synthesis with a nearly ten-fold increase in the concentration of hydrocortisone suggests heterogeneity in the cell population. This heterogeneity may reflect two non-mutually exclusive possibilities: variation in maturity in a single "type" of lymphocyte or the presence of more than one type.

DISCUSSION

The results of these experiments can be discussed with respect to *in vivo* information, and as *in vitro* phenomena.

Direct evidence for an *in vivo* alteration of immune response was seen with HCG and hydrocortisone. These will be considered in turn.

The reservations in attributing validity to the observed effect to HCG have already been pointed out. Since no *in vivo* experiments have been carried out with this hormone and we must rely entirely on observations concomitant with its natural occurrence, which in non-pathological states is pregnancy. The peak concentration, as already mentioned, is reached at about two months gestation and drops thereafter to a comparatively low value. If the *in vivo* effect of this hormone and the susceptibility and exposure of leukocytes were similar to that in tissue culture, then an association could be reasonably expected between the peak of HCG concentration and an alteration in circulating leukocytes. Insofar as numbers are concerned no association is evident.

It is, however, possible (but completely speculative) that associated with high HCG levels is the concomitant occurrence of a "protective agent." This is analogous to the protection given by an increase in levels of transcortin against the high hydrocortisone concentrations seen under similar circumstances.

Hydrocortisone has been shown to suppress the immune reaction in a number of species and this is likely also true in humans although conclusive evidence is lacking. As previously mentioned, high levels of hydrocortisone are characteristic of human pregnancy. There appears to be no profound effect of pregnancy on immunity in humans but there is an effect in rabbits (Heslop et al., 1954) which has been attributed to increased corticosteroid secretion. It is of interest that a significantly decreased absolute gamma globulin concentration is found in pregnant women (calculated from Brackenridge, 1964), however, this may be due to transfer to the fetus rather than a decrease in production. Nevertheless, lymphocytes are also decreased and are not transferred to the fetus.

No data are available on the effects of plasma from pregnant women on lymphocyte proliferation in vitro but the results presented suggest that such a difference should exist. With an adequate number of subjects each of the associations noted here could be critically tested with respect to their in vivo significance. This could be achieved by adding washed leukocytes from a single cell donor to cell-free media containing the appropriate plasma and PHA. This would have to be repeated throughout the gestation period. The peaks of plasma HCG and hydrocortisone concentrations are sufficiently different to ascertain the qualitative effects of each.

The action of hydrocortisone supports the relationship noted by Allison et al. (1965) in which substances which stabilize the lysosomal membranes also depress lymphocyte proliferation. The

reverse has also been noted, for example the ∝ toxin of Staphylococcus aureus decreases lysosomal stability and also induces lymphocyte proliferation in vitro.

The correlation with lysosomal stability is likely due to the similarity of the lysosomal and plasma membranes, with the latter serving as the initial site of action of this hormone. Thus Weissmann (1965a; Weissmann et al., 1965b), using steroids which varied in their stabilizing effect on rabbit membranes, found that plasma and lysosomal membranes responded similarly. Recently Herberman et al. (1965) demonstrated the presence of H-2 antigens of mice, which are classical plasma membrane components, on lysosomes.

The observed effect of hydrocortisone then would appear to be at the cell surface, since this is the first place of contact. It was demonstrated by Roseneau et al. (1962) that this hormone did not prevent the recognition of antigen since viable competent lymphocytes agglutinated with the target cell in its presence although the immune reaction did not progress. This was also demonstrated in an elegant experiment by Jennings et al. (1964) in which 10 μgm/cc of hydrocortisone prevented immune hemolysis of sheep cells but not the attachment of antibody. Such protected cells when transferred to antibody-free media, and dialyzed to remove the hormone, immediately lyzed. The effect of a synthetic steroid, Prednisolone, has a temporal sequence which suggests the effect as being on such a readily accessible site. Elves et al. (1964) found that if this hormone were added five minutes after PHA the result was a reduced ability to prevent lymphocyte proliferation.

On the other hand, progesterone has been found to decrease lysosome stability (Weismann, 1965a) and hence should increase lymphocyte proliferation *in vitro*. The negative result obtained in this study was not due to culture conditions inadequate to demonstrate this mode of stimulation (supposing it were different from that induced by PHA) since staphylococcal filtrate was highly active under the same conditions (see Appendix 16). This filtrate, prepared according to Ling *et al*. (1964) from *Staphylococcus aureus*, contains α toxin, a potent disruptor of lysosomes.

Likely the ineffectiveness of progesterone is due to its very high complexing activity with plasma proteins (Cope, 1964, p. 62). This was also demonstrated in experiments by Weismann *et al*. (1965b) in which a concentration of progesterone which was highly hemolytic was rendered inactive by the addition of a small amount of serum.

An association is apparent between the action of PHA, phagocytosis and the hormonal influence on the plasma membrane.

By analogy with known antigens PHA should be recognized by a lymphocyte whose plasma membrane was stabilized by hydrocortisone. However, this seems not to be an adequate stimulus for lymphocyte proliferation and an indispensible step appears to be the internal processing of the macromolecule; hence phagocytosis (or pinocytosis) would be required. That PHA is in some way associated with phagocytosis is suggested also by its toxic effect on monocytes (Schrek *et al*., 1964), which are normally highly phagocytic, perhaps as a result of excessive stimulation of an already well developed function.

As pointed out by Nicol *et al.* (1964) phagocytosis *in vivo* is inhibited by corticosteroids and enhanced by estrogens, a classification which bears some resemblance to that affecting plasma membrane stability (Weismann *et al.*,1965b). Certainly some of the exceptions to this assumed pattern could be due to experimental procedures such as the inhibition of progesterone action by serum which was present in one case but not the other. If this assumption is valid it provides strong evidence for a functional relationship between phagocytosis and membrane stability.

It would then seem that lymphocyte proliferation requires the phagocytosis of a macromolecule, a process dependent on its interaction with the cell membrane and the stability of this membrane, the latter property being greatly influenced by substances such as hydrocortisone.

SUMMARY

The previous section demonstrated the importance of plasma constituents in the modification of the immune reaction *in vitro*. It then seemed worthwhile to observe the effect of certain plasma constituents (hormones) on the immune response of human lymphocytes *in vitro*, using PHA activity as a model for immune stimulation. It was shown that progesterone at concentrations found normally in pregnancy did not affect lymphocyte proliferation and confirmation was also made of Mauer's (1963) report that 17-β estradiol at normal pregnancy levels was ineffective in altering the rate of proliferation. It was, however, observed that HCG at physiological levels significantly reduced lymphocyte proliferation. The lethal effect at high concentrations of preparations of this hormone suggested the presence of a toxic contaminant. Contrary to the work of Mauer (1963) hydrocortisone at high but nevertheless physiological levels reduced to a variable extent the rate of lymphocyte proliferation.

A suggestion was made that the mode of action of hydrocortisone is the inhibition of PHA uptake due to its apparent depressant effect on phagocytosis probably caused by the increased stability of the plasma membrane.

GENERAL SUMMARY

An association was demonstrated between human and avian mixed leukocyte cultures and the reaction in the latter, though not fully tested for technical reasons, was at least not inconsistent with the hypothesis which attributes the lymphocyte proliferation characteristic of these cultures to stimulation by histocompatibility isoantigens.

Human lymphocytes were shown to proliferate more slowly in the presence of a number of naturally occurring hormones. Lack of comparable *in vivo* data precludes a detailed comparison between lymphocyte proliferation and the immune response but the potential effect of these hormones on the latter was demonstrated.

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APPENDIX 1

Let

E_c = erythrocytes counted in smear of original culture suspension

L_1 = lymphocytes counted in smear of original culture suspension.

E_s = erythrocytes counted in smear after addition of known quantity of erythrocytes

and

L_2 = lymphocytes counted in smear after addition of known quantity of erythrocytes.

From the procedure outlined on page we obtain the ratios

(i) E_c/L_1

and (ii) $E_c + E_s/L_1$

Let

$$C = \frac{E_c/L_1}{(E_c + E_s)/L_2}$$

If

$$L_1 \neq L_2$$

let

$$L_1 = kL_2$$

then

$$C = \frac{E_c/L_1}{k(E_c + E_s)/L_1} = \frac{E_c}{k(E_c + E_s)}$$

therefore

$$CkE_c + CkE_s = E_c$$

and

$$CkE_s = E_c(1 - Ck)$$

and finally

$$\frac{E_s}{E_c} = \frac{1 - Ck}{Ck}$$

Since C and k are constants for each culture and E_s is of known concentration the concentration of E_c can be directly determined.

Summation gives the total concentration of erythrocytes/mm^3

The ratio (immature lymphoid cells/erythrocytes) directly gives the concentration of the former in the smeared suspension. Appropriate correction for dilution gives the culture value.

APPENDIX 2

Immature lymphoid cells in mixed chicken leukocyte cultures

Data transformed to $\sqrt{x + 1}$ (to allow use of ANOV for cell counts) where x is the number of immature lymphoid cells per 100 erythrocytes derived from the added erythrocyte suspension; this corresponds to 0.2 mm^3 of the original culture.

Cell origin	215	215 - 110	110
	1.41	2	1.73
	1	2.45	1
	1	2.24	1
	1	1.73	1.73
	1	1.41	1.41
	1.41	2	1
	1	2.65	1
	1	1.41	1

ANOV

Source	s.s.	d.f.	m.s.	F	F .05
Cell origin	3.64	2	1.82	15.2**	3.47
Error	2.50	21	0.12		
Total	6.14	23			

To compare the mixed culture to the average of the controls the method of Snedecor (1964, p. 255) was used. This gave F = 29.75** where F .05 = 4.32.

Chapter 2

Literature Review on Solar Energy Resource Assessment

Solar energy is the [illegible] energy source that is [illegible] in all countries across the world. It is the source of energy [illegible] life on earth. Solar energy can be used directly or indirectly [illegible] other renewable energy resources. The intensity of [illegible] at the earth's surface [illegible].

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APPENDIX 3

Hydrogen ion concentration (pH) of mixed chicken leukocyte cultures and controls

Cell origin	215	215 - 110	110
	7.4	7.0	7.1
	7.4	7.2	7.1
	7.4	6.8	7.2
	7.4	7.2	7.2
	7.3	7.0	7.2

ANOV

Source	s.s.	d.f.	m.s.	F	F .05
Cell origin	.30	2	.15	13.6**	3.89
Error	.13	12	.011		
Total	.43	14			

A comparison between the mixed cultures and the mean of the controls gave G = 16.0** when F .05 = 4.75.

APPENDIX 4

Mixed leukocyte reaction in chickens

The cell suspensions were as follows:

Bird No.	Geno.	Eryth.	Lymph.	Mono.	Granulo.	Thromb.	Total
110	B_2B_{14}	661	1810	35	139	835	3480
		19	52	1	4	24	100%
289	$B_{13}B_{13}$	343	1144	143	57	1173	2860
		12	40	5	2	41	100%

The heparin was preservative free; the media was 14% hen plasma in NCTC 109 (Baltimore Biological Associates).

A differential count was made on one replicate with the following results:

Culture	Lymphocytes counted	Immature lymphoid cells counted (% of total in brackets)	
110	320	4	(1.25)
289	388	0	(0.00)
110-289	454	15	(3.30)

The data are inadequate for statistical analysis. The expected percentage of immature lymphoid cells in the mixture (based on the relative frequency of each in the mixture and the proportion of immature lymphocytes found in each of the controls) is .77 percent.

The observed value is 4.29 times larger than expected.

The pH's of these cultures were:

110	110 + 289	289
7.5	7.3	7.4
7.6	7.4	7.5
	7.3	
	7.4	

The pH of the mixed culture is significantly lower than the average of the controls.

ANOV

		d.f.	m.s.	F	F .05
Treatment	45	1	45	9*	5.99
Error	30	6	5		
Total	75	7			

APPENDIX 5

DNA synthesis (cpm x 100) for a 12 hour period on day 5 in mixed human leukocyte cultures and controls

Cell origin	KTP	KTP + WTH	WTH
	66	45	75
	2	30	2
	2	128	7
	4	79	24
$\bar{x}$	18.5	70.5	27

ANOV

Source	s.s.	d.f.	m.s.	F	F .05
Cell origin	6,225	2	3113	2.34	5.14
Block	4,049	3	1350	1.02	4.76
Error	7,969	6	1328		
Total	18,243	11			

A comparison between the mixed cultures and the mean of the controls gave F = 4.58 where F .10 = 3.78 and F .05 = 5.99.

APPENDIX 6

Hydrogen ion concentration (pH) of mixed human leukocyte cultures and controls (Appendix 4) at time of harvest

Cell origin	KTP	KTP + WTH	WTH
	7.1	7.0	7.2
	7.1	7.1	7.2
	7.1	7.0	7.2
	7.1	7.1	7.2
$\bar{x}$	7.10	7.05	7.20

ANOV

Source	s.s.	d.f.	m.s.	F	F .05
Cell origin	.05	2	.025	22.72**	4.26
Error	.01	9	.0011		
Total	.06	11			

A comparison between the mixed cultures and the mean of the controls gave F = 27.27** where F .05 = 5.12.

APPENDIX 7

DNA synthesis (cpm x 100) for a 12 hour period on day 6 in mixed human leukocyte cultures and controls

Cell origin	KTP	KTP + WTH	WTH
	29	122	2
	50	159	3
	26	179	84
$\bar{x}$	35	153.3	29.6

ANOV

Source	s.s.	d.f.	m.s.	F	F .05
Cell origin	29,324	2	14,662	17.54**	6.94
Block	3,100	2	1,550	1.85	6.94
Error	3,344	4	836		
Total	35,768	8			

A comparison between the mixed cultures to the mean of the controls gave F = 35.0** where F .05 = 7.71.

APPENDIX 8

Hydrogen ion concentration (pH) of mixed human leukocyte cultures and controls (Appendix 6) at time of harvest

Cell origin	KTP	KTP + WTH	WTH
	7.1	7.1	7.2
	7.1	7.0	7.2
	7.1	7.0	7.2
$\bar{x}$	7.10	7.03	7.20

ANOV

Source	s.s.	d.f.	m.s.	F	F .05
Cell origin	.04	2	.02	12.5**	5.14
Error	.01	6	.0016		
Total	.05	8			

A comparison between the mixed cultures and the mean of the controls gave F = 18.8** where F .05 = 5.99.

APPENDIX 9

Effect of HCG (30 iu/cc) on DNA synthesis (cpm x 100) in each culture

Block	Period of synthesis (hrs)					
	Experimental			Control		
	36 - 48	48 - 60	60 - 72	36 - 48	48 - 60	60 - 72
1	101	222	290	204	415	582
2	96	193	476	167	364	766
3	73	210	409	147	534	492
4	77[a]	88	268	189	477	588
5	29	79	79	160	304	490
6	46	50	277	119	267	405
$\bar{x}$	70.3	140.3	300	164	393.5	553.8

ANOV

Source	s.s.	d.f.	m.s.	F	F .05
Treatment	361,401	1	361,401	89.08**	5.12
Block	122,477	5	24,495	6.04*	3.48
Period	574,955	2	287,478	70.86**	4.26
Treatment x block	21,457	5	4,291	1.06	3.48
Treatment x period	50,935	2	25,468	6.28*	4.26
Block x period	79,455	10	7,946	1.96	3.14
Error	36,516	9	4,057		
Total	1,247,196	34			

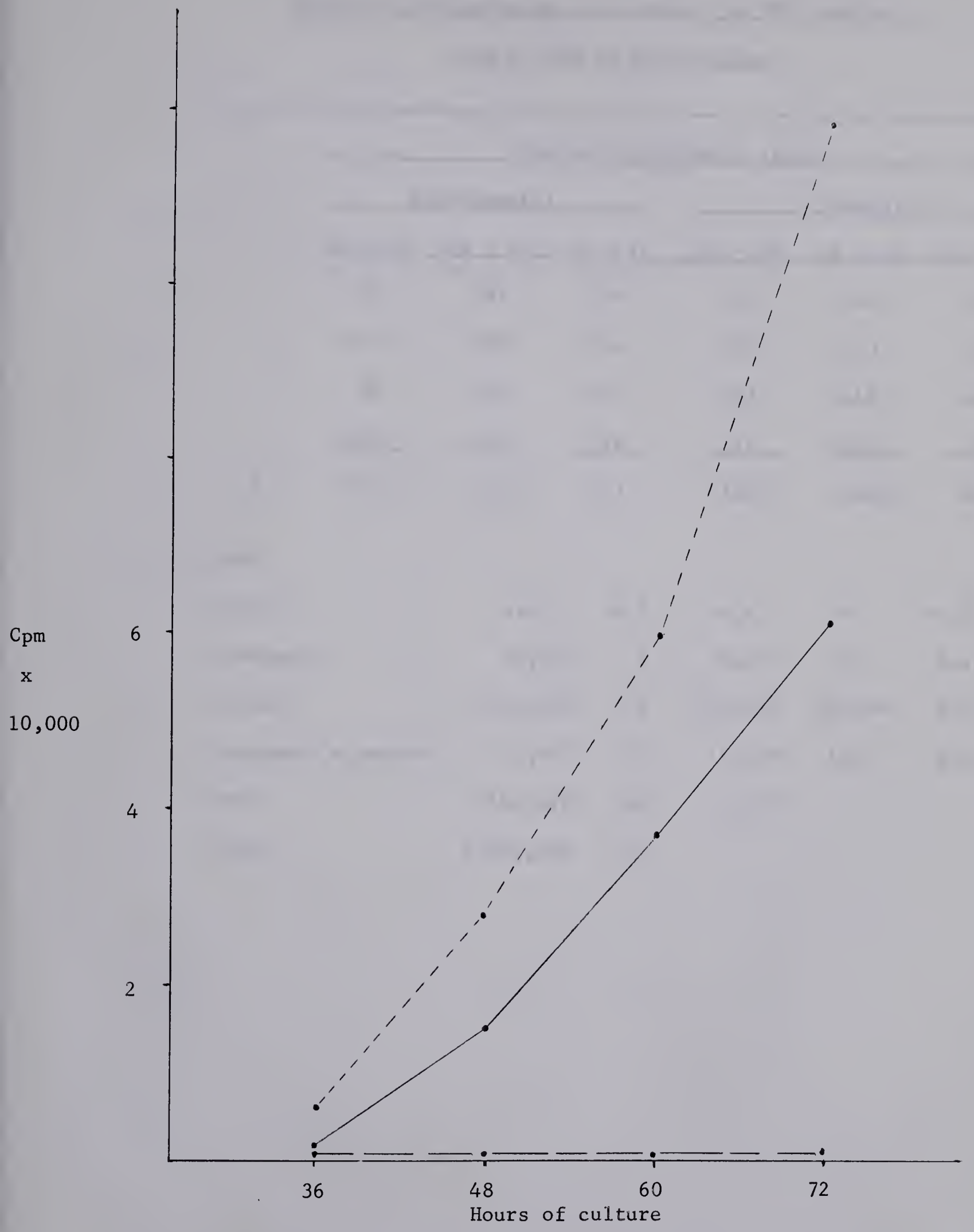
[a] Missing data, estimated according to Snedecor (1964, p. 310).

APPENDIX 10

Effect of HCG on DNA synthesis

The cultured cell suspension consisted of

Eryth.	Lymph.	Mono.	Granulo.	Total
2594	714	38	414	3760
69	19	1	11	%



Effect of HCG (iu/cc) on DNA synthesis. Each point (all single culture determinations except those at 72 hours each of which is the mean of two cultures) represents the synthesis in a 12 hour period added to the mean of the preceding periods (starting at hour 24) to give a cumulative measure of DNA synthesis (HCG 300 iu — —; HCG 30 iu ____; control ----).

APPENDIX 11

Effect of progesterone (0.1 μgm/cc) on DNA synthesis (cpm x 100) in each culture

	Period of synthesis (hrs)					
	Experimental			Control		
	36 - 48	48 - 60	60 - 72	36 - 48	48 - 60	60 - 72
	170	383	738	130	328	571
	260	466	760	157	333	767
	88	432	775	191	448	681
	183	134	779	76	333	476
$\bar{x}$	175.3	353.8	763	138.5	360.5	623.8

ANOV

Source	s.s.	d.f.	m.s.	F	F .05
Treatment	19,097	1	19,097	2.3	4.41
Period	1,175,990	2	587,995	70.8**	3.55
Treatment x period	22,477	2	11,239	1.35	3.55
Error	149,498	18	8,305		
Total	1,367,062	23			

APPENDIX 12

Effect of progesterone on DNA synthesis

The cultured cell suspension consisted of:

Eryth.	Lymph.	Mono.	Granulo.	Total
2130	512	0	544	3200
67	16	0	17	100%

Results

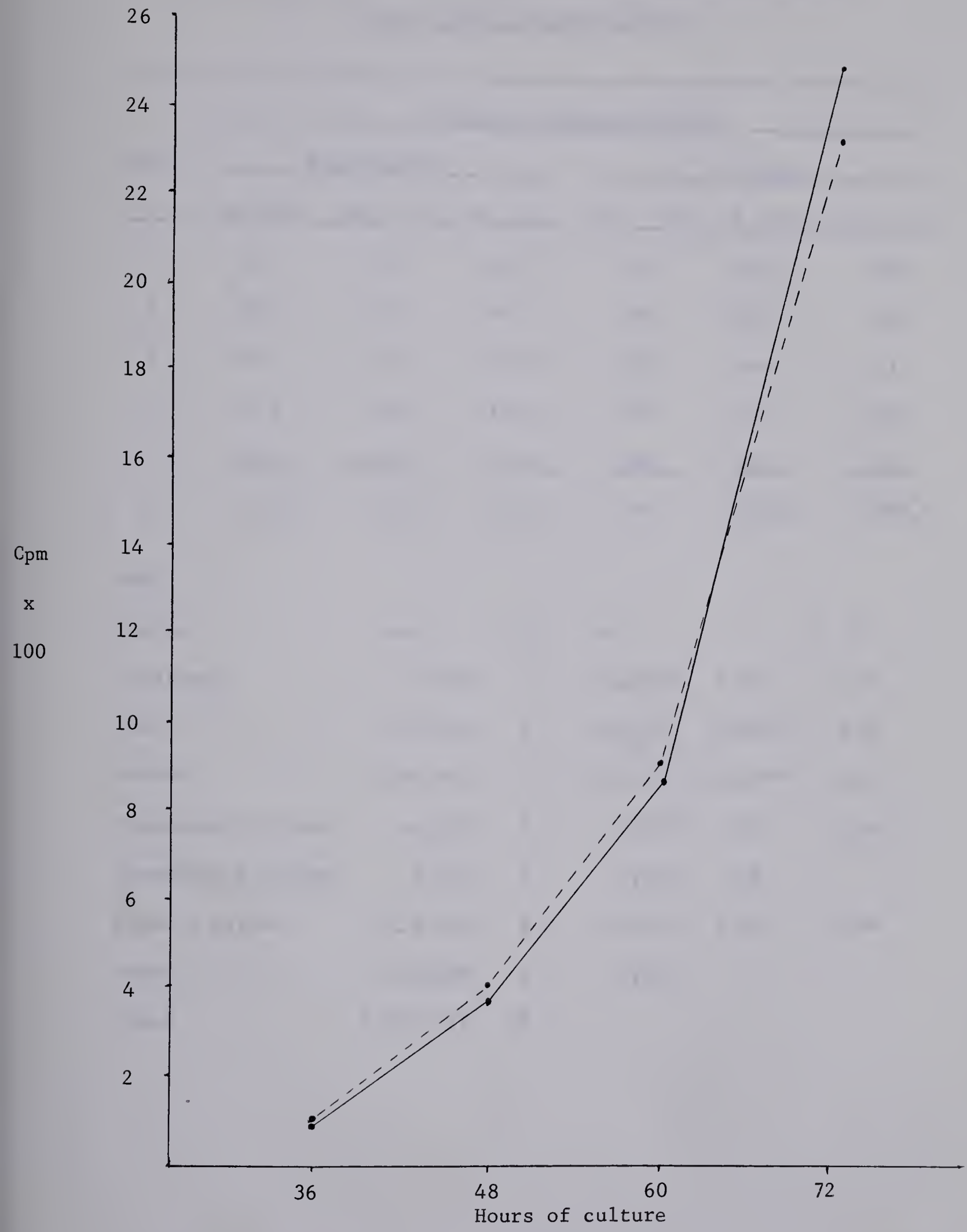
Period of synthesis hrs. (Cpm x 100)

	24 - 36	36 - 48	48 - 60	60 - 72
Exp.	10	30	64	120
	7	23	33	123
	13	29	56	251
$\bar{x}$	10	27.3	51	163.6
Control	10	26	22	118
	12	29	51[a]	106
	9	36	80	204
$\bar{x}$	10.3	30.3	51	142.6

[a] Estimate for missing value.

ANOV

		d.f.	m.s.	F	F .05
Treatment	131	1	131	.10	4.54
Period	73,835	3	24,612	19.23**	3.29
T x P	609	3	203	.16	3.29
Error	19,203	15	1,280		
Total	93,778	22			



Effect of progesterone (0.1μgm/cc) on DNA synthesis. Each point is the mean of 3 cultures for a 12 hour period added to the mean of the preceding periods (starting at hour 24) to give a cumulative measure of DNA synthesis (Progesterone ——; control ----).

APPENDIX 13

Effect of 17-β-Estradiol (0.01 μgm/cc) on DNA synthesis (cpm x 100) in each culture

Block	Period of synthesis (hrs)					
	Experimental			Control		
	36 - 48	48 - 60	60 - 72	36 - 48	48 - 60	60 - 72
1	347	514	1277	372	530	1499
2	290	527	1619	399	520	1523
3	301	443	1353	289	494	1211
4	243	346	1063	201	363	1188
5	172	263	740	269	342	1127
$\bar{x}$	270.6	418.6	1210.4	306	449.8	1309.6

ANOV

Source	s.s.	d.f.	m.s.	F	F .05
Treatment	22,908	1	22,908	2.93	5.32
Block	433,392	4	108,344	13.85**	3.84
Period	5,491,445	2	2,745,723	350.89**	4.46
Treatment x block	44,889	4	11,222	1.43	3.84
Treatment x period	7,260	2	3,630	.46	
Block x period	158,816	8	19,852	2.54	3.44
Error	62,601	8	7,825		
Total	6,221,311	29			

APPENDIX 14

Effect of 17-β estradiol on DNA synthesis

The cultured cell suspension consisted of:

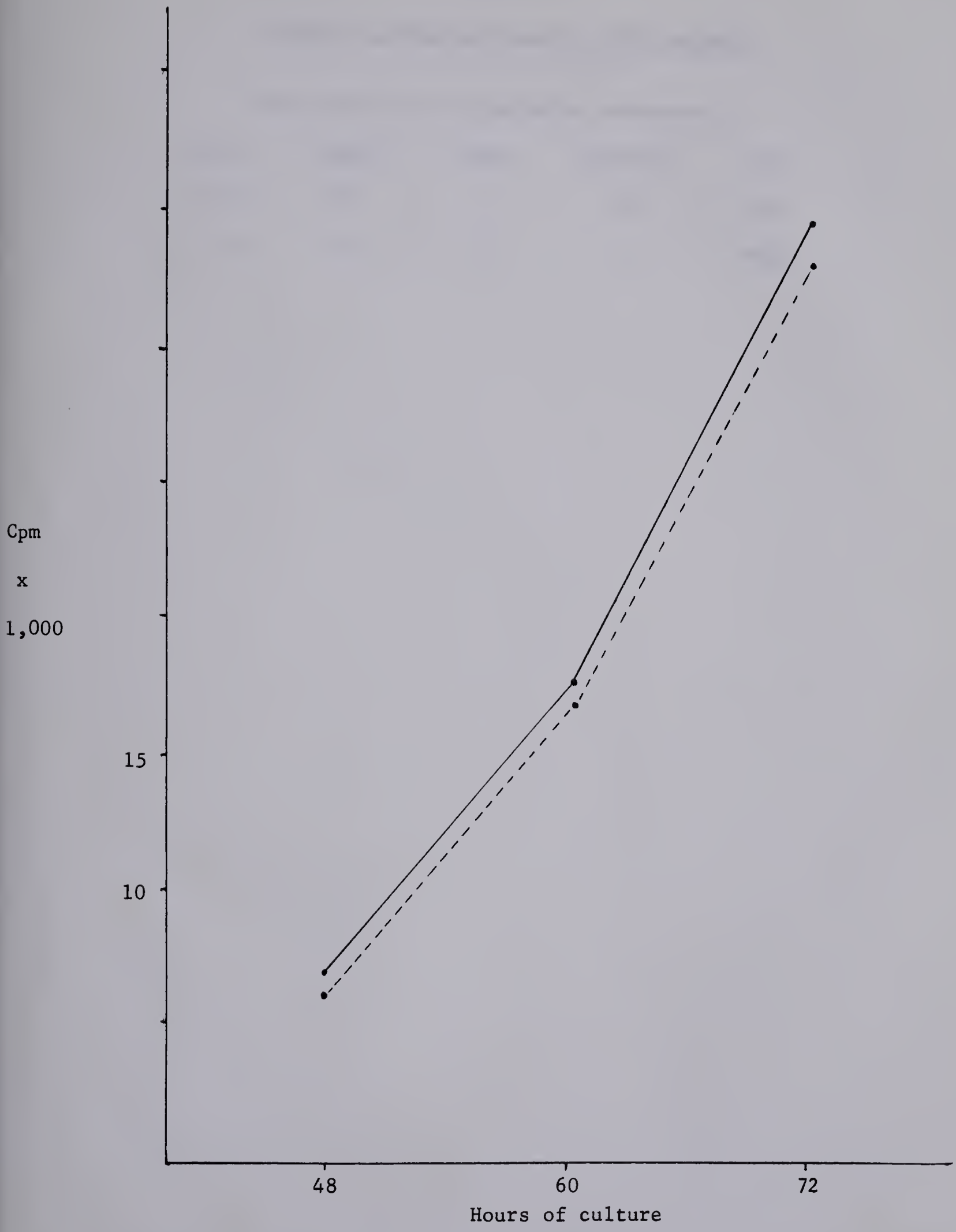
Eryth.	Lympho.	Mono.	Granulo.	Total
1300	374	40	276	1970
66	19	2	13	100%

	Experimental			Control		
	36 - 48	48 - 60	60 - 72	36 - 48	48 - 60	60 - 72
	64	105	192	60	103	155
	75	114	174	62	124	175
	74	103	151	61[a]	96	162
$\bar{x}$	71	107	172	61	108	164

ANOV

		d.f.	m.s.	F	F .05
Treatment	162	1	162	1.10	4.84
Period	31,682	2	14,841	107.76**	3.98
T x P	92	2	41	.28	3.98
Error	1,620	11	147		
Total	33,556	16			

[a] Estimate for missing value.



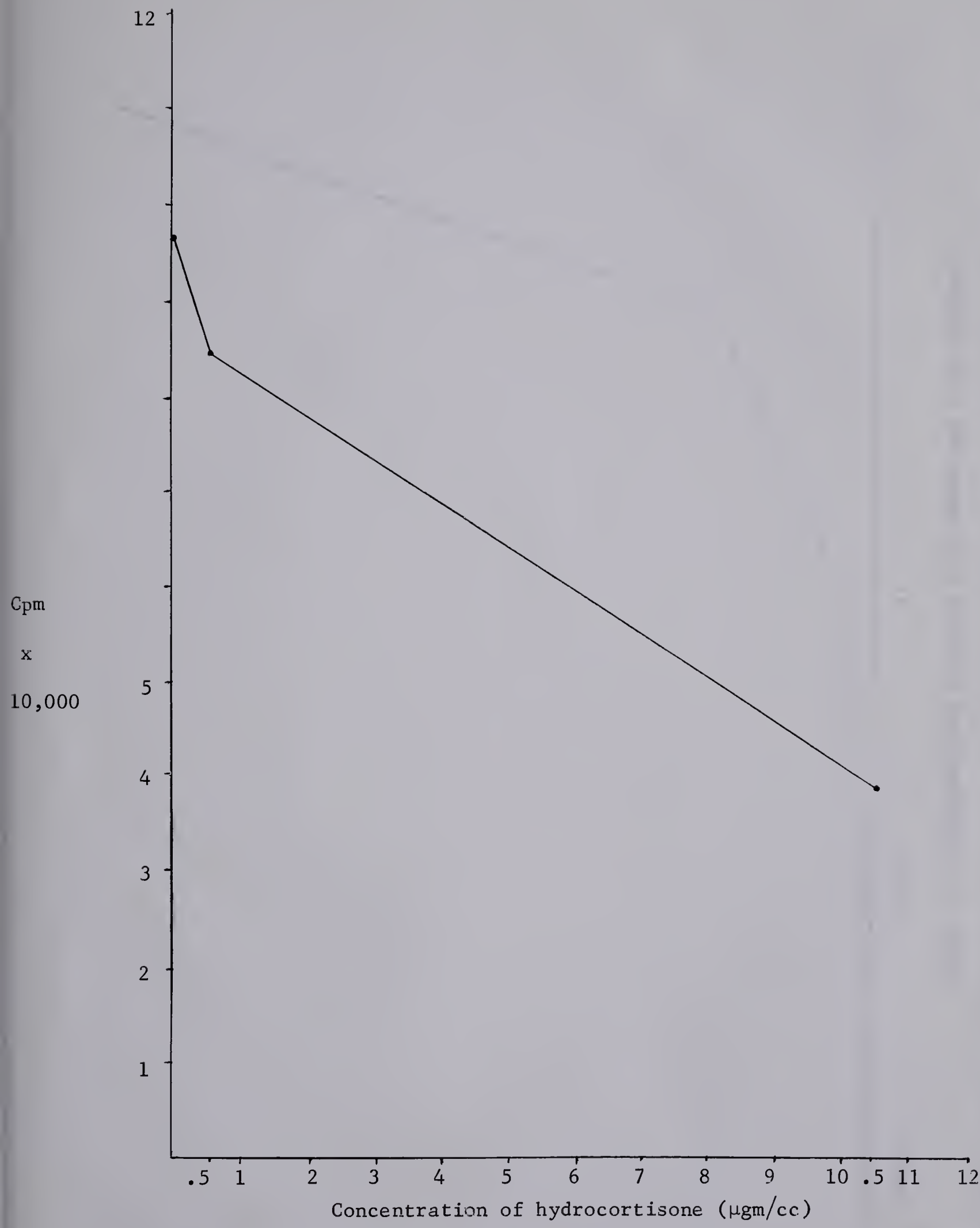
Effect of 17-β estradiol on DNA synthesis. Each point is the mean of 3 cultures for a 12 hour period added to the mean of the preceding periods (starting at hour 36) to give a cumulative measure of DNA synthesis (Estradiol ——; control ----).

APPENDIX 15

Effect of hydrocortisone on DNA synthesis

The cultured cell suspension consisted of:

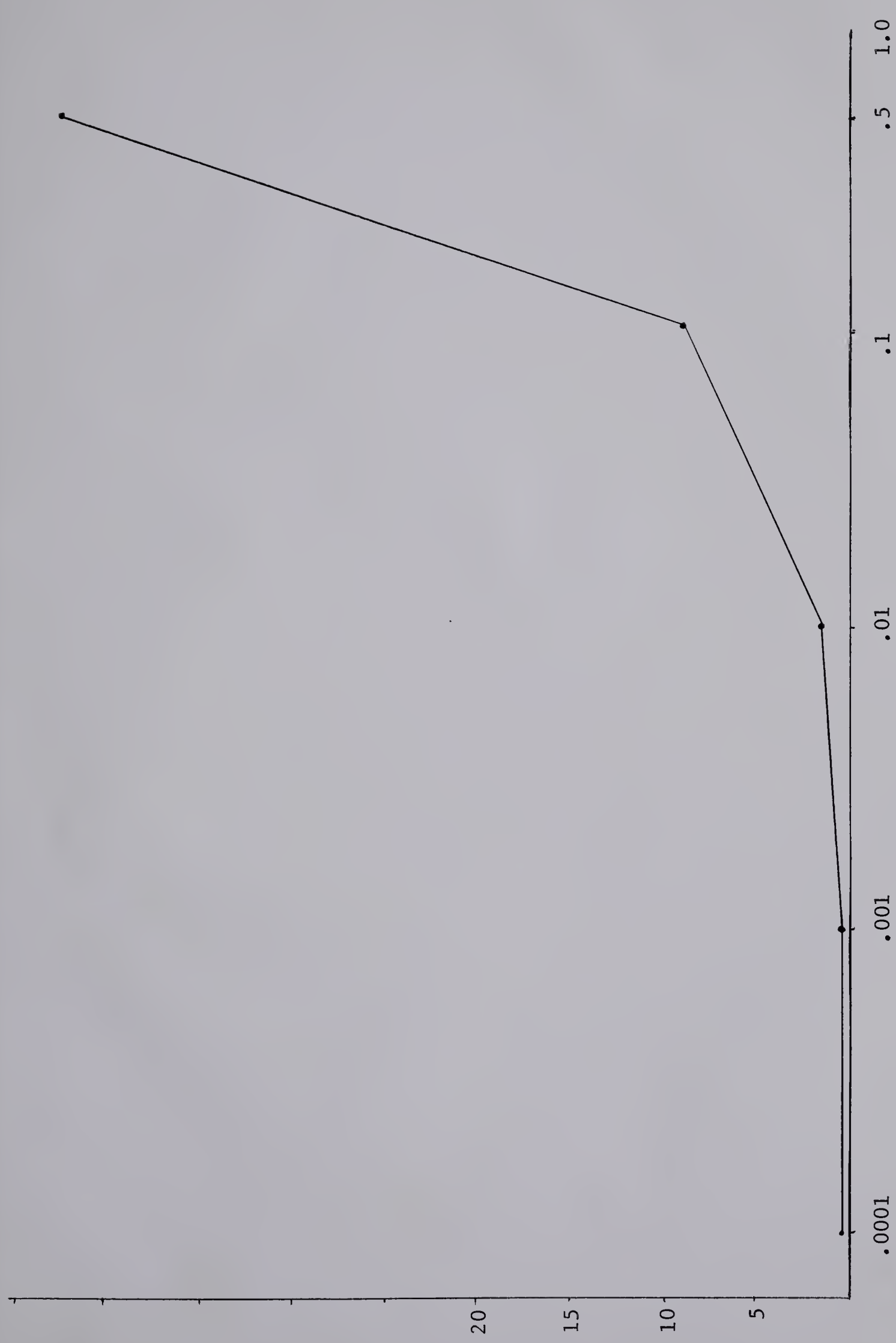
Eryth.	Lymph.	Mono.	Granulo.	Total
1390	331	0	119	1840
76	18	0	6	100%



Effect of various concentrations of hydrocortisone on DNA synthesis for a 12 hour period (60 - 72 hours). (Each point is the mean of 2 cultures except that for 0.5 µgm level which is the mean of 3 cultures.)

APPENDIX 16

Effect of staphylococcus filtrate on DNA synthesis



The strain used was 42-D derived from the Department of Microbiology, University of Alberta. The control gave 397 cpm. All points are single determinations except that for 0.1 level which is the mean of two cultures.